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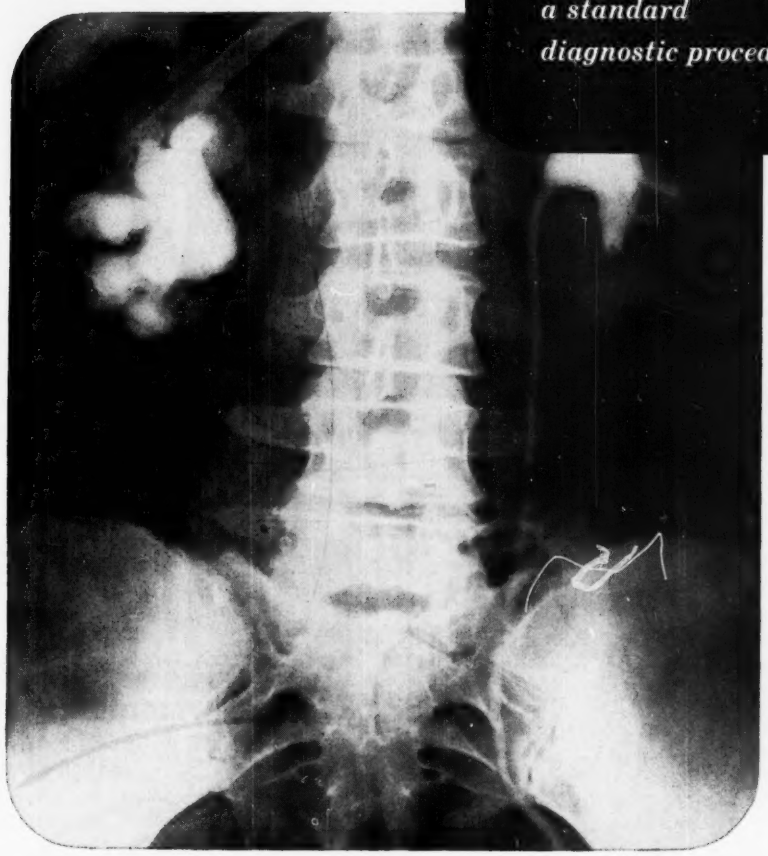

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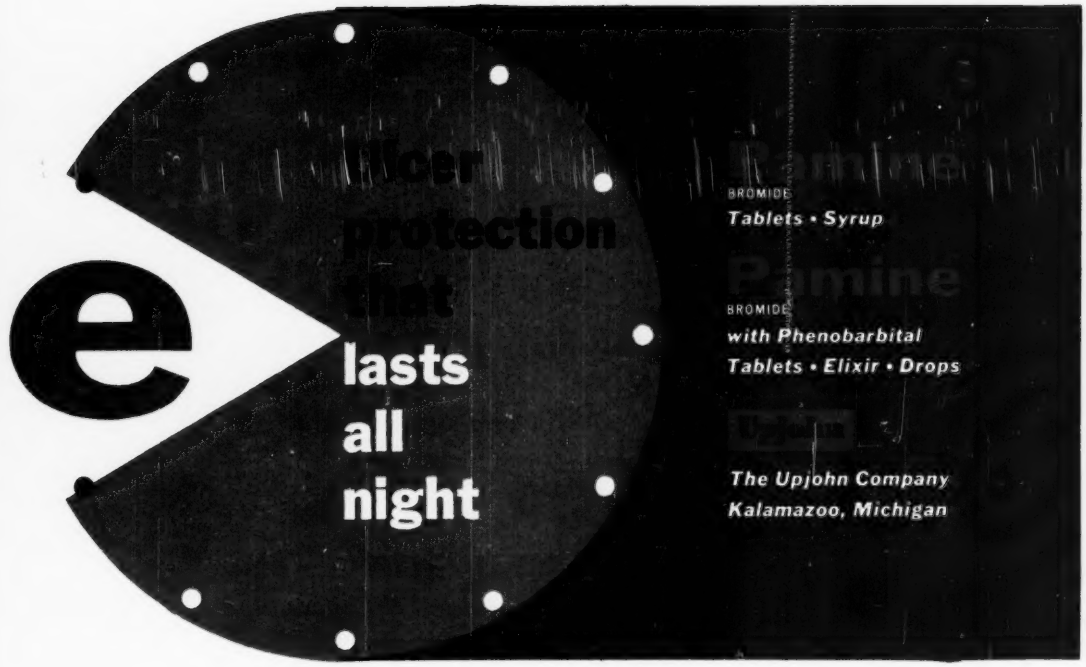

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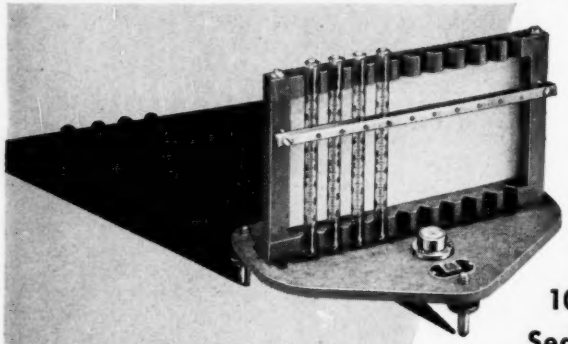

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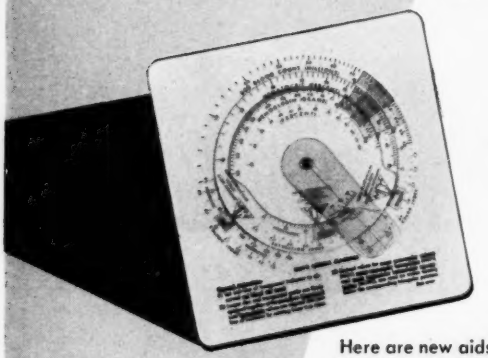

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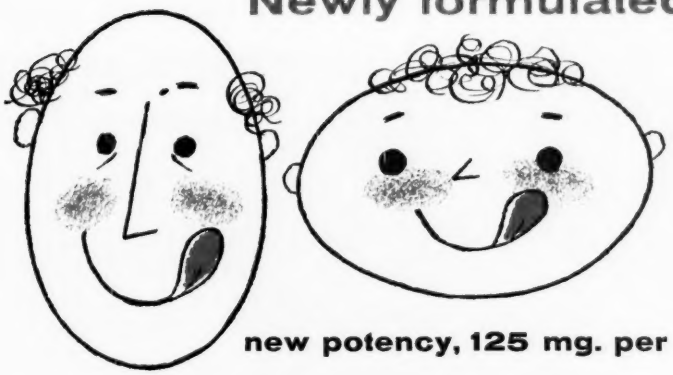

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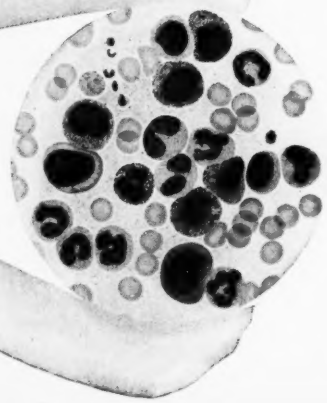

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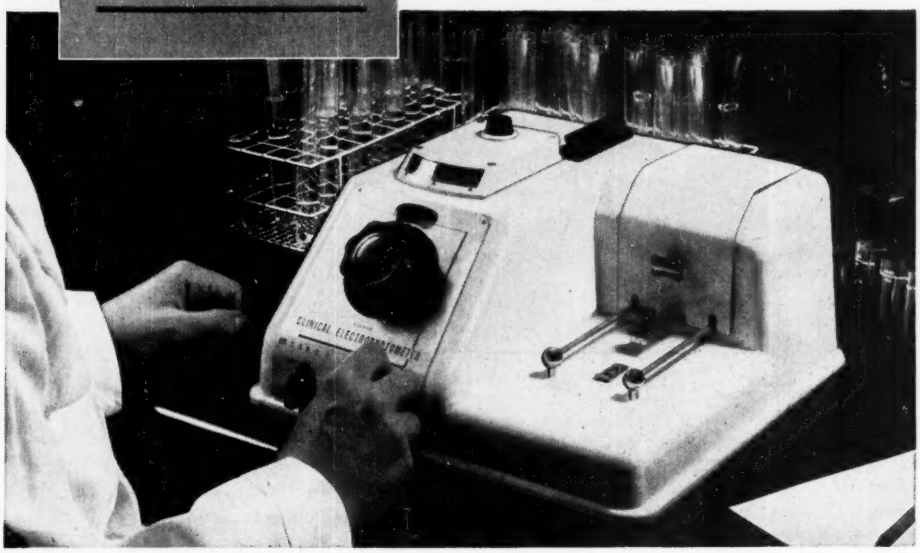

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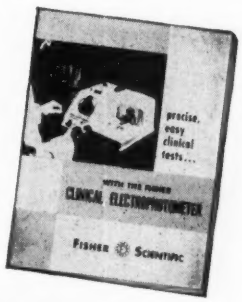

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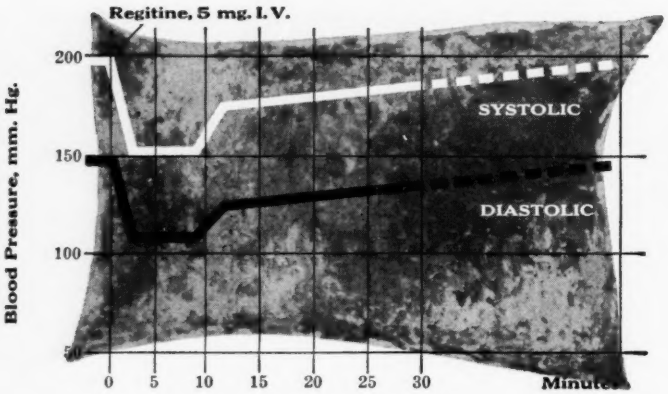

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PROTEINS AND OTHER BIOCOLLOIDS OF URINE IN HEALTH AND IN CALCULOUS DISEASE. I. ELECTROPHORETIC STUDIES AT pH 4.5 AND 8.6 OF THOSE COMPONENTS SOLUBLE IN MOLAR SODIUM CHLORIDE [1]

By WILLIAM H. BOYCE, FRED K. GARVEY, AND CHARLES M. NORFLEET, JR.

(From the Department of Urology, Division of Surgery, Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N. C.)

(Submitted for publication March 4, 1954; accepted June 16, 1954)

In approximately 92 per cent of all primary urinary calculi, more than 95 per cent of the total cation content is composed of calcium (1–3). Concepts concerning the mechanism of biologic calcification have undergone a marked revision in recent years (4). Rubin and Howard (5) have presented histochemical evidence that an acid mucopolysaccharide of cartilage and bone matrix may act as a "target substance" for the deposition of calcium and the eventual formation of bone salts. The structure of urinary concretions suggests that the initial matrix may be composed of ion-binding mucopolysaccharides or other "substrates," which not only provide the architectonic framework but may actually "extract" specific inorganic components of urine from solution. The separation and identification of the organic components of urine are essential prerequisites to an exploration of this possibility.

A series of experiments, in which some subjects have been repeatedly examined over a period of two years, has demonstrated that the biocolloids of urine may be separated into three groups of substances, on the basis of their solubilities in different buffer systems. It is the purpose of this report to present the results of studies on one of these groups—those components which are soluble in molar sodium chloride and in two buffers which have been used in the electrophoretic study of serum proteins (6–8).

METHODS

Three female and 10 adult male subjects from the staff of the North Carolina Baptist Hospital and the Bowman Gray School of Medicine were selected as a control group; all were known to have normal renal function. The test group was made up of 17 patients (7 females and 10 males) selected over a period of 20 months because they were demonstrated roentgenographically to have progressively developing calculi. Later analysis of these stones showed them to contain pure calcium phosphate or a mixture of calcium phosphate and calcium oxalate. These patients were studied prior to operation, and no individual with signs or symptoms of acute infection was included. In addition, two patients, ages 7 and 11 years, with the nephrotic syndrome and two patients, ages 16 and 18 years, with lupus erythematosus were studied as examples of altered capillary permeability. Four additional patients with bilateral obstructive urinary tract disease and bacterial pyelonephritis were studied before operation, but after the signs of pyelonephritis had subsided.

Clean voided specimens of urine were collected into chemically clean containers without preservatives, and immediately refrigerated at 0° C. Hospitalized subjects were provided with bedside ice chests to insure prompt refrigeration. Each 24-hour specimen was centrifuged at 800 times gravity to remove formed elements, and then dialyzed against distilled water at 3° C. until the electrical conductivity approached 100×10^{-6} and the pH was the same as the distilled water.

The dialyzed specimens were subjected to ultrafiltration in stainless steel filters through a modification of the collodion membranes described by Gorbman (9). Variations in porosity of the membranes were minimized by preparing 30 individual membranes from the same collodion mixture. These membranes, prepared on glass plates floated on mercury with exact control of reagents, temperature and time, were of a reproducible porosity, as judged by the filtration rates for distilled water and retention of Bence-Jones protein. The membranes were stored in distilled water to which a few thymol crystals were added, and urine specimens from at least one normal subject were examined with membranes from each batch.

Ultrafiltration was conducted in the cold room at 3 to 5° C. Forty-eight hours was required for filtration of a normal 24-hour specimen through a single membrane under increasing pressures of 5 to 125 pounds per square inch. The large quantity of colloidal material contained

[1] This investigation was supported by research grant A-259 from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, Public Health Service.

in the urine of patients with calculi frequently required three to six membranes for filtration of a 24-hour specimen.

The colloidal residues were removed from the membranes as a gel, immediately frozen, and stored until a total of 72 to 240 hours of continuous urinary output per subject had been processed. To insure removal of all soluble colloids from the membranes, each was shredded and immersed in 30 ml. of Veronal buffer, ionic strength 0.1, pH 8.6, and stored at 1.0° C. In a preliminary series of experiments the membranes were dissolved in 15 ml. of ethanol-ether at 0.5° C.; the precepitate was then extracted three times with ethanol-ether, dried with ether, and added to the solid gel obtained above. This procedure did not appear to have any advantage over buffer extraction of the membranes.

After completion of the collection period, each 24-hour colloidal residue was extracted three times with 10 ml. of the buffer used to extract the collodion membrane, and finally with 10 ml. of fresh buffer. Extraction with molar sodium chloride gave results which were quantitatively and qualitatively similar to the buffer extracts from the same individuals. All of the soluble proteins contained in the gel seemed to carry a high concentration of adsorbed or bound urinary pigments. These pigments served as a convenient guide to the completeness of extraction. The pooled extracts of normal urine usually totaled 120 to 200 ml. per 72 to 120 hours of urinary excretion. These were concentrated to a volume of 8 ml. by pervaporation—that is, suspension of the solution in cellophane bags 15/16 inch wide in a cold air stream at 5° C. Similar results have been obtained by pressure dialysis against 2 liters of Veronal buffer, and this technique has been routinely used for subsequent experiments.

The total protein content of a small sample of the dialyzed extract was determined by the biuret method, and the solution was diluted with Veronal buffer to give a resulting protein content of 1 per cent. A final equilibration dialysis against Veronal buffer of pH 8.6 was made prior to electrophoretic studies with the Aminco electrophoresis unit and calibrated cell of 8 ml. capacity. The proteins were allowed to migrate for 7200 seconds under a potential gradient of 7.4 volts per cm. at 0.5° C. in Veronal buffer of ionic strength 0.1 and pH 8.6 (10). Photographs were made with the slit diaphragm, cylindrical lens system, using a red filter and high contrast panchromatic film.

Samples of urinary proteins from the same patients were also studied electrophoretically at pH 4.5, by the method others (8, 11–14) have used for serum proteins. After the proteins had been collected and concentrated in Veronal buffer, they were dialyzed against an acetic acid, sodium acetate buffer of pH 4.5, ionic strength 0.1 (15) for 72 hours. Intermediate dialysis against phosphate buffers to prevent precipitation was found to be unnecessary. Electrophoretic migration was studied in the same cell for three to five hours under a potential gradient of 6.0 volts per cm. at 0.5° C.

A very small precipitate which formed during the acid dialysis was removed by centrifugation. This precipitate was much larger in the group of patients with calculous disease than in the control group. It could not be completely redissolved in Veronal buffer of pH 8.6, but the soluble portions from five patients and from three normal subjects were studied electrophoretically in Veronal buffer, pH 8.6, ionic strength 0.1, at a potential gradient of 7.4 volts per cm. for two hours. The insoluble portion gave a positive Molisch test and was soluble in glycine buffer of pH 10.

RESULTS

The colloidal gel obtained from normal 24-hour specimens measured 0.90 to 1.25 ml. in volume. The dry weights of these lyophilized residues from five normal subjects averaged 0.0902 Gm. per 24 hours, with a range of 0.0865 to 0.0975. In the patients with calculous disease the lyophilized residue averaged 0.496 Gm. per 24 hours, with a range of 0.296 to 1.253.

Following extraction with Veronal buffer there was a considerable residue of gel-like material, which occupied a volume almost as great as the original material. After lyophilization, the dry weights of these residues from five normal subjects were in the range of 0.0439 to 0.0448 Gm. per 24 hours. On the basis of dry weights it appears that approximately 42.6 to 52.7 mg. of the original gel was extracted by Veronal buffer. The ratio of the insoluble residue to the total colloids was much higher in the group of patients with calculi. Since no component of this gel was soluble in Veronal buffer of pH 8.6 or in acetate buffer of pH 4.5, without procedures which modify its molecular structure, further studies of this material will not be considered in the present report. The largest single component of this residue is a conjugate of carbohydrate and protein, which has been subjected to electrophoretic, spectroscopic, and biochemical analysis. The resulting data are being prepared for publication.

Electrophoretic studies at pH 8.6

Figure 1 illustrates the most typical Schlieren pattern obtained from normal urine, as compared with the maximal variations encountered in 18 determinations from 13 normal subjects. These variations are in accord with the observations of Rigas and Heller (16), who noted minor variations in the patterns obtained from different individuals, whereas the pattern of a given individual tended to remain constant over several months.

In Figure 2 are shown tracings of patterns obtained from normal plasma, as compared with tracings of the patterns made by urinary proteins subjected to electrophoretic studies at pH 8.6. A comparison of Figures 2-A and 2-B reveals that all the electrophoretically separable protein fractions found in normal serum are also present in normal urine. This observation was originally made by Rigas and Heller (16), who identified as a mucoprotein the component with a mobility greater than that of albumin. These investigators also identified an immobile polysaccharide as the cause of the unusually large delta and epsilon boundaries. We have observed a tendency for these "salt plus polysaccharide" boundaries to show a very slight migration toward the cathode. Since the mobilities of the albumin fractions have been consistently reproducible in all of these studies, the projected patterns were aligned on the albumin peaks rather than on the delta and epsilon boundaries. The illustration is thus more nearly representative of the calculated mobilities as measured from the initial boundary. There is evidence that fibrinogen does not occur even in nephrotic urine (7, 17–19), and the small boundary near the position of theta may be properly designated gamma-1 globulin.

The pattern illustrated in Figure 2-C was obtained from the same subject and during the same 10-day period of collection as the pattern seen in Figure 2-B. Alternate 24-hour specimens were subjected to the technique of recovery already described, with the result shown in Figure 2-B. The remaining five days' collection was concentrated by the alcohol-ether precipitation method described by Rigas and Heller (16), with the result shown in Figure 2-C. Two components with mobilities greater than albumin (fc-1 and fc-2) are present in both. Figures 2-D, E and F illustrate the proteins present in the urine of patients with various diseases of the urinary tract.

Comparable patterns obtained by the electrophoretic study of proteins in the serum and urine of a patient with lupus erythematosus are shown in Figure 3. All of the Veronal-soluble proteins in a single 24-hour specimen of urine, 420 ml. in volume, were concentrated into 10 ml. of buffer, and the resulting protein solution of 3.5 per cent was subjected to electrophoresis.

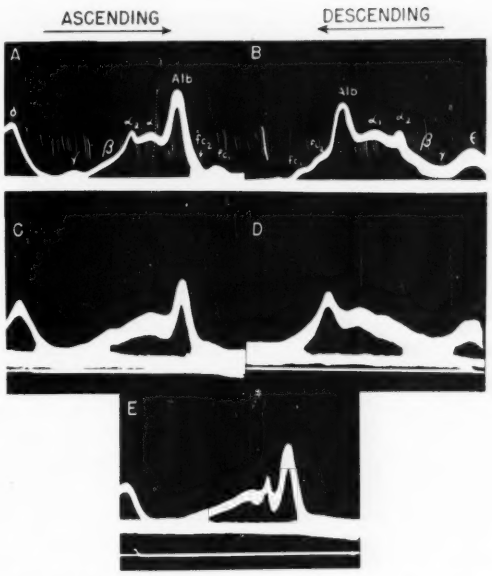

FIG. 1. SCHLIEREN DIAGRAMS OF URINARY PROTEINS FROM NORMAL SUBJECTS

A and B are the most typical patterns encountered in 13 subjects. C, D and E represent the greatest variations from the typical normal pattern encountered in 18 determinations.

Figure 4 shows the electrophoretic patterns obtained from normal urine and from the urine of five patients with calculous disease. These five patterns are typical of the variations from the normal which were observed in all of the 17 patients with renal stones. As measured by the biuret test, the average protein excretion in this group was 0.204 Gm. per 24 hours with a range of 0.086 to 0.312. The patterns of all of these patients were markedly similar, and were characterized by a separation of the albumin from the adjacent large boundaries.

As determined by calculations after the method of Dole (6), the mobilities of the albumin and of the beta and gamma globulin fractions in the urine of these patients were comparable to those in normal urine and normal serum. The mobilities of the two boundaries between the albumin and beta globulin peaks were essentially the same for each of the patients with calculous disease, but varied significantly from those of the alpha-1 and alpha-2 fractions of normal urine. These two boundaries

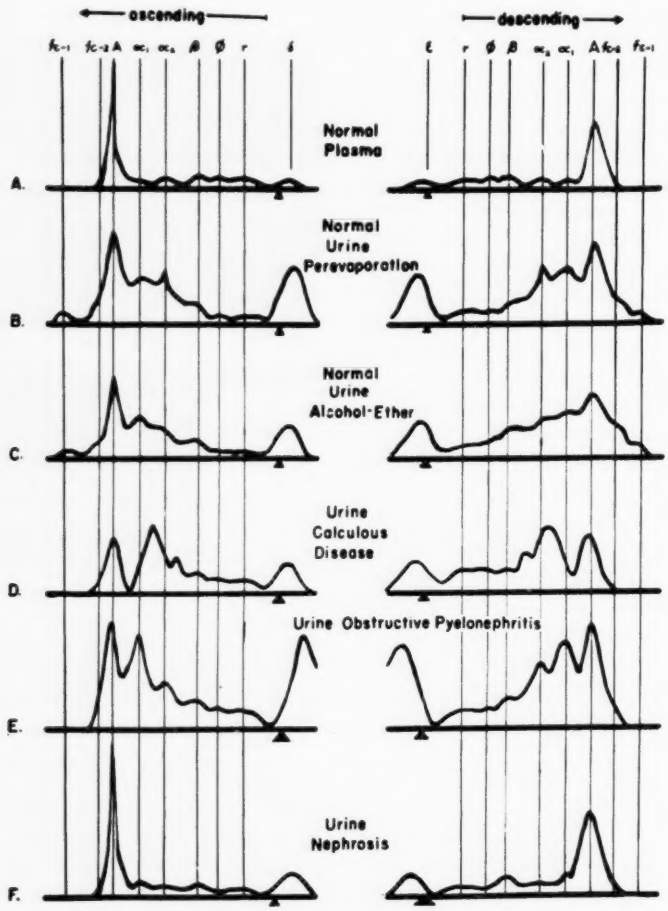

FIG. 2. TRACINGS OF ELECTROPHORETIC MIGRATION PATTERNS OF NORMAL HUMAN PLASMA AND OF URINARY PROTEINS IN HEALTH AND IN THREE DISEASE STATES ACCOMPANIED BY INCREASED PROTEINURIA

Veronal buffer, pH 8.6, ionic strength 0.1, potential gradient 7.4 volts per cm. for 7,200 seconds.

Tracings of the projected patterns are utilized to eliminate inequalities of exposure which the presence of urinary pigments introduces into the original photographs. These inequalities do not impair the usefulness of any single pattern, but preclude the simultaneous copying of the large number of patterns used in this illustration.

together comprised 46.7 per cent of the total amount of protein in the urine of patients with calculous disease (Table I). The larger peak had an average mobility of $-4.81\ (\pm 0.11) \times 10^{-5}$ cm.^{2}/volt sec., and the smaller an average of $-3.92\ (\pm 0.02) \times 10^{-5}$ cm.^{2}/volt sec. Dole (6) gives a mobility of -5.07×10^{-5} cm.^{2}/volt sec. for alpha-1 globulin and -4.08×10^{-5} cm.^{2}/volt sec. for alpha-2 globulin.

In Figures 4-B, 4-C and 6-B a small peak can be seen between those representing albumin and the large components of calculous disease urine just discussed. These small peaks have a calculated mobility of the same value as alpha-1 glob-

ulin of the serum. This fact suggests that the alpha globulins are present in calculous disease urine, but may be "overshadowed" by the large volume of those components which are thought to be mucoproteins.

In Table I the components of the urinary proteins in normal subjects and in 25 patients with various diseases, as measured in planimeter units by the method of Longsworth, are averaged and expressed as percentages of the total. In Table II the approximate average quantities of these components are expressed as milligrams per 24 hours of urinary excretion.

Electrophoretic studies at pH 4.5

Figure 5 shows the patterns obtained when urinary proteins and serum proteins from the same normal subject were studied by electrophoresis at pH 4.5. Under these conditions the albumin forms a stationary boundary, while the globulins are positively charged and migrate toward the cathode. The serum globulins separate into three components—A, B and C—which have been demonstrated to represent, respectively, the alpha, beta and gamma globulin fractions observed at pH 8.6 (12, 13). The acid mucoprotein MP-1 (orosomucoid) seen in normal serum has not been conclusively demonstrated in normal urine, but has seemed to be present in minute concentrations in some specimens. An acid component with the same mobility as MP-2 was always present (Figure 5).

A precipitate formed during the acid dialysis of the proteins, and only a very small portion could be redissolved in Veronal buffer of pH 8.6. The Veronal-soluble portion gave a positive biuret tests. When it was subjected to electrophoretic studies, only albumin could be detected, although the electrophoretic patterns in Figure 4 indicate a possible loss of globulin also.

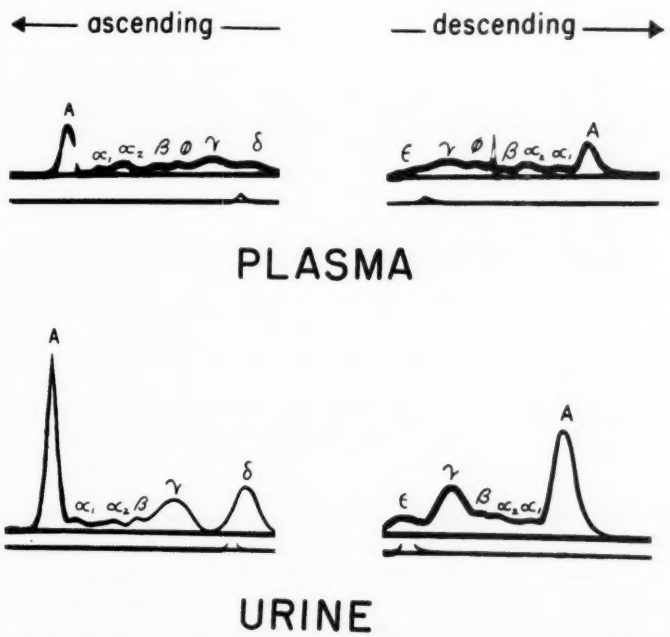

FIG. 3. ELECTROPHORETIC MIGRATION PATTERNS OF PROTEINS IN THE PLASMA AND URINE OF A PATIENT WITH LUPUS ERYTHEMATOSUS

Veronal buffer, pH 8.6, ionic strength 0.1, potential gradient 7.4 volts per cm. for 7,200 seconds.

The albumin peak of the ascending pattern of the urinary proteins extended beyond the photographic plate, and has been drawn into the print.

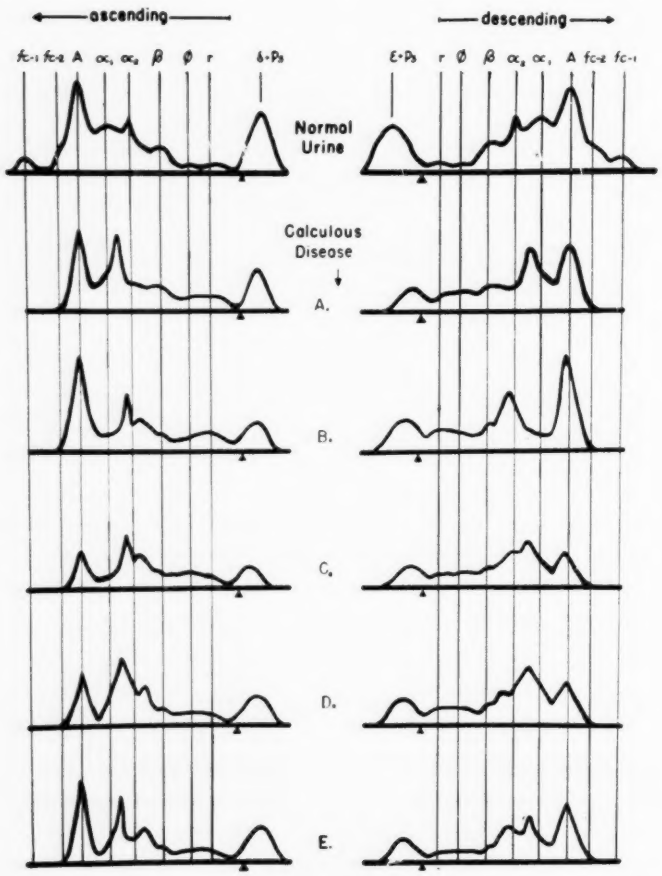

FIG. 4. TRACINGS OF ELECTROPHORETIC MIGRATION PATTERNS OF URINARY PROTEINS IN HEALTH AND IN CALCULOUS DISEASE

Veronal buffer, pH 8.6, ionic strength 0.1, potential gradient 7.4 volts per cm. for 7,200 seconds.

In a preoperative study of the patient with a functioning adenoma of the parathyroid gland (4-C), a 24-hour specimen of urine contained 180 mg. of proteins.

In Figure 6 the electrophoretic patterns from normal urine and from the urine of patients with calculous disease and chronic pyelonephritis are compared at pH 8.6 and 4.5. For the purpose of this illustration the proteins were recovered from the cell following studies at pH 8.6, concentrated to the original volume, and dialyzed to a pH of 4.5. The precipitate which occurred in every case was composed primarily of a polysaccharide (Molisch test) which was immobile at pH 8.6. In the normal and pyelonephritic urines the only other detectable component of the precipitate was a very small amount of albumin. In the urine of patients with calculous disease the precipitate contained a relatively greater concentration of the mucoprotein than of albumin (Figure 6-G). Note that, although the precipitated albumin retains the normal mobility for albumin at pH 8.6, the mobility of the precipitated mucoprotein is even less than it was in the original determination at pH 8.6

TABLE I

Relative concentrations of proteins in normal serum, normal urine, and the urine of patients with renal calculi and other pathologic conditions

Subjects	Determinations	Quantity mg./24 hrs.	A/G Ratio	Area of each boundary determined by planimetry and expressed as % of total						
				fc-1	fc-2	Albumin	Alpha-1	Alpha-2	Beta	Gamma
Normal serum*		7.23 Gm./100 cc.	1.33			56.8	7.2	8.7	12.8	14.4
Normal urine (13)	18	42.6 to 52.7	0.64	2.2	?	37.9	27.3	19.5	8.8	3.3
Calculous disease urine (17)	36	86 to 312	0.45			28.8	28.4	18.3	7.3	17.2
Obstructive pyelonephritis urine (4)	6	263	0.37			26.9	36.2	18.1	8.8	10.1
Nephrosis urine (2)	4	800 to 1,610	1.94			66.0	7.5	8.5	10.4	7.5
Lupus erythematosus urine (2)	3	350	1.15			53.6	1.5	6.0	5.7	33.2

* Reiner, M., Fenichel, R. L., and Stern, K. G., Electrophoretic studies on the protein distribution in normal human serum. Acta Haematologica, 1950, **3**, 202.

(Figure 6-B). This material was recovered from the cell and gave both a positive Molisch test and a positive biuret test.

The unprecipitated mucoprotein of calculous disease urine contributes to the stationary albumin boundary, but in both the normal and the pyelonephritic urines the mucoprotein migrates toward the anode. This finding has been generally true in patients with calculous disease, although the presence of a small quantity of negatively charged mucoprotein in the urine of some of these patients has been demonstrated at pH 4.5.

DISCUSSION

The Schlieren diagrams are of necessity illustrated on the same scale. For this reason, anyone not thoroughly familiar with the techniques involved may be deceived regarding the concentration of protein in the individual urine specimens. For example, the pattern for normal urine in Figure 4 represents the total proteins from 8.1 liters of urine (120 hours' output), whereas the pattern shown as Figure 4-A represents the total proteins from only 1.6 liters of urine (20 hours' output). The purpose of Tables I and II is to show the relative quantities of the urinary proteins per 24 hours of collection time. The figures in these tables are not presented as absolute values, but they are of considerable importance in the interpretation of the Schlieren diagrams.

These figures were obtained by determining the lyophilized dry weight of the total colloidal residue of one 24-hour urine specimen from each subject. The soluble proteins were extracted from this residue with 0.1 molar Veronal buffer of pH 8.6, by the method used in preparing samples for the electrophoretic studies. The lyophilized dry weight of the remaining insoluble residue was then determined, and the difference in weights was taken as the value for the soluble proteins. The individual components were determined by planimetry, and the area of each component was translated into milligrams on the basis of the difference in dry weights.

TABLE II

Average quantity of urinary proteins obtained from normal subjects and from patients with renal calculi and other pathologic conditions

Subjects	Determinations	Milligrams per 24 hours of urinary excretion						
		fc-1	fc-2 Albumin	Alpha-1	Alpha-2	Beta	Gamma	Total
Normal (13)	5	0.9	17	12	9	4	2	45
Calculous disease (17)	17		59	58	37	15	35	204
Obstructive pyelonephritis (4)	2		70	95	48	23	27	263
Nephrosis (2)	2		620	71	80	98	71	940
Lupus erythematosus (2)	2		187	5	24	19	115	350

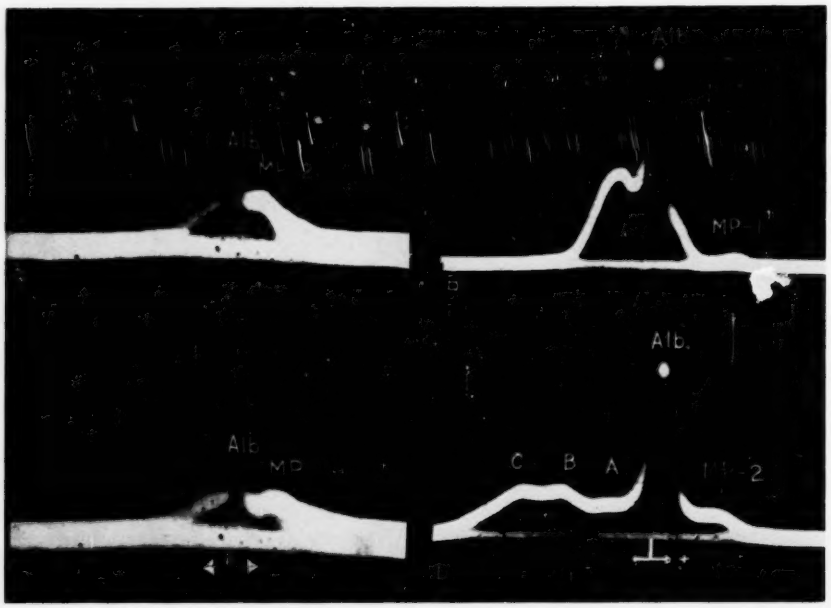

FIG. 5. ELECTROPHORETIC MIGRATION PATTERNS OF NORMAL SERUM AND OF NORMAL URINARY PROTEINS AT PH 4.5

Slit diaphragm, cylindrical lens patterns of boundaries ascending toward the anode. Acetate buffer, pH 4.5, ionic strength 0.1, potential gradient 6.2 volts per cm.

A. Urinary proteins at 3,600 seconds.
B. Serum proteins at 3,600 seconds.
C. Urinary proteins at 10,800 seconds.
D. Serum proteins at 10,800 seconds. MP-1 has migrated beyond the photographic range.

Certain errors are obviously inherent in this technique. The polysaccharide, which contributed to the total weight of the soluble material, is immobile at pH 8.6 and is not included in the planimetric studies, since a variable portion of this refractive gradient (E + Ps in Figure 4) is also due to the epsilon "salt" boundary. Inspection of the patterns will demonstrate that planimetric separation of the individual gradients is much more arbitrary in studies of these urinary proteins than in comparable studies of serum proteins. Furthermore, the overlap of fc-2 and albumin gradients made it desirable to express these values as a single sum (Tables I and II).

There are two components of normal urine whose electrophoretic mobilities at pH 8.6 are greater than that of albumin. The urinary mucoprotein recovered by Tamm and Horsfall (20) is electrophoretically homogeneous at pH 6.8 and 8.6 (21), and has a mobility slightly greater than that of human serum albumin (22). This mucoprotein is insoluble in molar sodium chloride, but it is possible that it may have a limited solubility in the 0.1 molar Veronal buffer used for extraction in our experiments. The refractive gradient fc-2 may therefore represent the mucoprotein of Tamm and Horsfall. Since these authors obtained a yield of 0.025 Gm. of mucoprotein per liter of urine, it is obvious that fc-2 (Figures 1, 2, 6) is only a portion of the total quantity of this mucoprotein present in the 72- to 120-hour urine specimens represented by these Schlieren patterns. This finding would be in accord with the limited solubility of this material in 0.1 and 0.025 molar buffers (20).

The large alpha globulin boundaries in normal urine at pH 8.6 are composed not only of the alpha globulins of serum but also of an acid protein. This protein has a mobility at pH 4.5 and

8.6 similar to that of the mucoprotein MP-2 previously observed in normal and pathologic human sera (8, 12–14). The greater portion of the alpha globulin boundaries of the urinary proteins is made up of this acid mucoprotein; and if it is taken into consideration in calculations of the albumin-globulin ratio of the urinary proteins, the result is a urinary A/G ratio even greater than that of normal serum.

Nephrosis and lupus erythematosus are diseases characterized by increased capillary permeability and increased proteinuria. The additional protein in the urine is composed almost entirely of proteins found in the serum of these patients. The lipoproteins found in the serum of nephrotic patients do not appear in the same relative proportions in the urine (19), but beta globulin is present in the urine (7). The gamma globulin fractions found in the blood of patients with lupus erythematosus do appear in the urine of these patients in increased concentrations. In these patients the urinary proteins have an A/G ratio more nearly similar to that of normal serum than to that of their own serum; this finding indicates a relatively greater urinary loss of albumin than of globulin.

Renal calculous disease is accompanied by increased proteinuria, presumably the result of inflammatory or exudative processes within the collecting portions of the urinary system. The urinary A/G ratio is reversed from that of normal

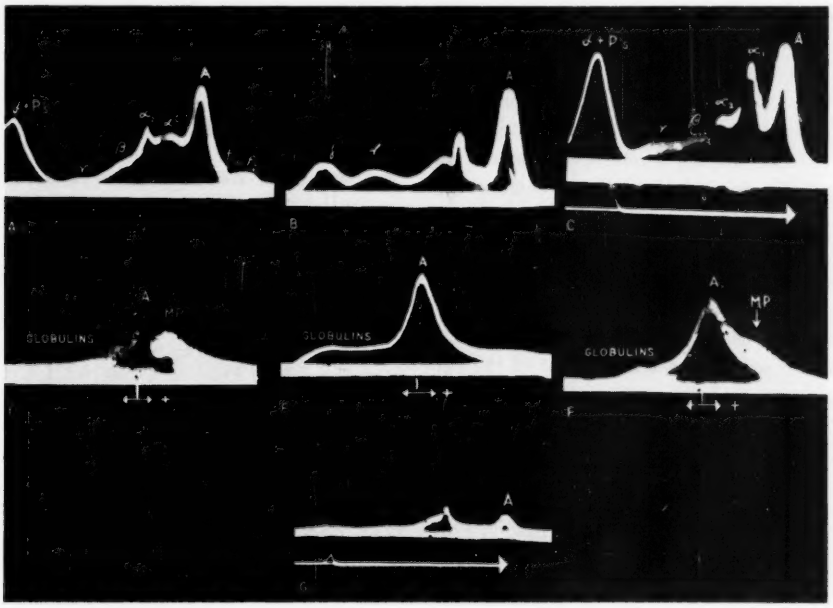

FIG. 6. ELECTROPHORETIC MIGRATION PATTERNS OF URINARY PROTEINS AT pH 8.6 AND pH 4.5

Patterns at pH 8.6 were taken after 7,200 seconds migration in Veronal buffer, pH 8.6, ionic strength 0.1, potential gradient 7.4 volts per cm. Patterns at pH 4.5 were taken after 10,800 seconds migration in acetate buffer, pH 4.5, ionic strength 0.1, potential gradient 6.0 volts per cm. All patterns are ascending toward the anode.

A. Normal urinary proteins at pH 8.6.
B. Calculous disease urinary proteins at pH 8.6.
C. Pyelonephritis urinary proteins at pH 8.6.
D. Normal urinary proteins at pH 4.5.
E. Calculous disease urinary proteins at pH 4.5.
F. Pyelonephritis urinary proteins at pH 4.5.
G. Precipitate from acid (pH 4.5) dialysis of calculous disease urinary proteins redissolved in Veronal buffer of pH 8.6.

serum, because the urine contains two components which have mobilities slightly less than the alpha globulins. These two components are isoelectric at pH 4.5, where they tend to precipitate spontaneously and to contribute to the stationary albumin boundary. Whether these are structural modifications of the alpha fractions of normal urine or are entirely different substances peculiar to the urine in calculous disease remains at present unknown. The observed alteration in the mobility of these alpha boundaries (Figures 2 and 4) may be due to the fact that, in patients with calculous disease, the relative concentrations of these and other components of urine differ from those found in normal subjects (Table II). Studies are now in progress to isolate and identify further these components from both normal urine and the urine of patients with calculous disease.

Obstruction of the urinary tract, with resultant bacterial infection, may persist in some individuals for prolonged periods of time without the formation of calculi. The electrophoretic patterns of the urinary proteins from such patients contain the negatively charged component (MP-2) at pH 4.5, and are more similar to the patterns of normal urine than to those of patients with calculous disease.

The presence of calculi in the urinary tract is not considered to be the cause of the abnormalities observed in the urinary proteins. Four of the patients in this series have been examined one to three times at intervals of 3 to 14 months after surgical removal of all the calculi. In every case the abnormalities observed in the preoperative examinations were present in the follow-up examinations. It is recognized, however, that such patients have a predisposition to recurrent calculus formation, and the presence of developing or unrecognized stones cannot be excluded at the present time.

SUMMARY

The total quantity of proteins and other particles of colloidal size in the urine of patients with calculous disease is 3 to 13 times greater than that in normal urine. Electrophoretic studies of those components soluble in buffers of pH 4.5 and 8.6 have demonstrated consistent variations in the proteins from the urine of patients with calculous disease as compared with normal urine. These variations have been most apparent in the "alpha" fractions.

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ERRATUM

A Possible Fourth Plasma Thromboplastin Component, by Theodore H. Spaet, Paul M. Aggeler, and Beverly G. Kinsell, J. Clin. Invest., 1954, **33**, 1095. Table V.

The item, "5% normal plasma + PTA-deficient blood" should read "5% normal plasma + PTC-deficient blood."

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EFFECT OF INTRAVENOUS INFUSIONS OF ETHANOL UPON ESTIMATED HEPATIC BLOOD FLOW IN MAN [1]

By ALBERT I. MENDELOFF

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(Submitted for publication April 15, 1954; accepted June 16, 1954)

During the course of studies concerning the utilization by the human liver of parenteral feeding solutions containing ethanol, it was observed that these infusions resulted consistently in changes in the estimated hepatic blood flow. The amount of ethanol infused into these human subjects produced blood ethanol concentrations far below those known to be intoxicating (1), and lower than those levels reported to achieve some degree of postoperative sedation (2). Since even massive ethanol dosage is reportedly without effect on mammalian hemodynamics (3), it seemed unlikely that the changes in estimated hepatic blood flow observed here were due to gross changes in the circulation. This report demonstrates that the infusion of small amounts of ethanol in saline into unanesthetized adult human subjects produces consistently significant increases in estimated hepatic blood flow, most probably as a result of a specific decrease in splanchnic peripheral resistance.

METHODS AND PROCEDURES

The subjects were young adult males with either gastric or duodenal ulcers, but without evidence of primary liver disease, malnutrition, anemia, or dehydration. On the morning of the study each was fasting (except for subject M. S., who had eaten a piece of toast) at 7:30 a.m., when he received a subcutaneous injection of 60 mg. sodium phenobarbital. At 8 a.m. each was subjected to hepatic vein catheterization (4). Following a priming dose of 150 mg. of the dye, an intravenous infusion of 0.85 per cent sodium chloride solution containing enough bromsulfalein (BSP) to produce an infusion rate of approximately 3 mg. per min. per square meter of surface area was begun. The actual amount of this "control solution" infused varied from 5 to 9 ml. per minute among the five subjects, but was maintained constant in each subject. After approximately 30 minutes, without changing the tubing or drip control, a second bottle was substituted for the first; this second bottle contained the same amount of BSP dissolved in the salt solution, to which absolute ethanol had been added in an amount sufficient to give a final concentration of 5 per cent w/v. This "alcohol solution" was then allowed to run in at approximately the same rate as had the "control solution" for periods varying from 50 to 80 minutes.

Blood samples were taken from peripheral veins and the right hepatic venous tree in all cases. In certain subjects arterial samples were also obtained from the radial or femoral arteries. These blood samples were analyzed in the following ways:

BSP concentrations were determined in serum by the method of Bradley, Ingelfinger, Bradley, and Curry (4) utilizing a Coleman, Jr. spectrophotometer; total reducing sugars were analyzed for by the method of Somogyi as modified by Nelson (5); lactic acid was determined by the method of Summerson and Barker as modified by LePage (6), and pyruvic acid by the method of Liu as modified by Friedemann and Haugen (7). Plasma pepsinogen was calculated in terms of micrograms tyrosine released per ml. of whole blood according to the technique of Mirsky, Futterman, Kaplan, and Broh-Kahn (8). The methods developed by Stotz, Westerfeld and Berg were used to determine the whole blood content of acetaldehyde (9) and of ethanol (10).

Two additional studies were carried out. In the first of these BSP in saline solution was administered to one subject for 90 minutes; at thirty-minute intervals the infusion rate was changed, so that he received the solution at rates of 20, 9, and 8 ml. per minute, respectively, for each thirty-minute period, representing 11, 4.95, and 4.4 mg. BSP per minute. These three rates constitute extremes beyond the range of variation in volume of solution administered per minute during the studies with the ethanol solution, and serve to demonstrate the effect upon EHBF of volume of solution and amount of BSP administered. In the second study, an infusion of a saline solution was administered to a healthy adult male at a rate of 8 ml. per minute for 20 minutes; at this point ethanol in saline was administered at exactly the same rate for 45 minutes, at the end of which time the original solution was again infused at the same rate. Cardiac output by the dye dilution method (11) was measured twice during the first saline period, three times during the ethanol period, and once during the second saline period. Results were expressed as the cardiac index—cardiac output in liters per minute divided by the surface area of each subject. Arterial and peripheral venous blood levels of ethanol were determined during the ethanol period.

[1] This work was carried out under Contract No. DA-49-007-MD-410 between the Office of the Surgeon General, Department of the Army, Washington, D. C., and the Washington University School of Medicine.

RESULTS

The data relating specifically to estimated hepatic blood flow may be seen in Table I. Here it will be noted that in every one of these five subjects there was a significant increase in estimated hepatic blood flow during the infusion of ethanol in saline. At the same time, the peripheral venous concentrations of BSP were either unchanged or slightly decreased, indicating that no impairment in the ability of the liver to remove dye had occurred, if one presupposes that extrahepatic removal of dye under these circumstances remained constant.

Data pertaining to the oxidation of ethanol, production or utilization of lactic acid and of pyruvic acid, and to the changes in blood glucose and plasma pepsinogen are summarized in Table II. The glucose levels were reasonably constant, as were the values for plasma pepsinogen; this constancy may be interpreted to mean that none of the subjects became unduly anxious during the carrying out of the procedures. The blood ethanol levels were in all subjects well below the concentrations known to be associated with symptoms of intoxication, and with one exception were below the levels found by Rice, Orr, and Enquist necessary to provide postoperative patients with a sedative and analgesic effect (70 to 100 mg. per 100 ml.) (2). All subjects showed an uptake of ethanol by the splanchnic system, presumably by the liver, although in E. R. this was much less than in the other four men, who demonstrate uptakes varying from 15 to 50 per cent of the peripheral concentrations. The changes in lactic acid concentrations evident in these studies showed no unidirectional features; the blood levels of this metabolite were generally elevated during the alcohol period, but there were marked variations in the concentrations in the hepatic vein blood. Pyruvate levels also varied considerably, tending to fall during the alcohol period, with lower concentrations consistently found in hepatic as compared to peripheral blood.

The study summarized in Table III was designed to demonstrate that the infusion rates employed in these studies do not change the estimated hepatic blood flow as the result of fluid added to the circulation. The experimental results summarized in Table IV show clearly that ethanol

TABLE I

Data used for calculating estimated hepatic blood flow (EHBF) in five subjects receiving i.v. saline in "control period" and i.v. saline + 5 per cent ethanol in "alcohol period" *

Subject		Minutes after infusion began	BSP mg./100 ml. serum P	BSP mg./100 ml. serum H	BSP mg./100 ml. serum P−H	BSP infused mg./min.	BSP removed mg./min.	100 R / P−H	1 / 1−Hct.	EHBF ml./min.	Per cent change from control
L. S.	Control	20	1.10	0.35	0.75	7.3					
	period	28	1.15	0.40	0.75	7.3	7.2	960	1.85	1,770	
	Alcohol	30	0.85	0.45	0.40	5.5					
	period	60	0.88	0.45	0.43	5.5	5.4	1,280	1.85	2,380	34.5
J. C.	Control	16	3.45	2.30	1.15	6.55					
	period	21	3.35	2.35	1.00	6.55	6.05	568	1.57	895	
	Alcohol	30	3.8	3.00	0.80	6.62					
	period	48	4.2	3.50	0.70	6.62	6.00	860	1.57	1,340	49.7
E. T.	Control	20	1.05	0.45	0.60	7.35					
	period	25	1.05	0.45	0.60	7.35	6.85	1,142	1.72	1,960	
	Alcohol	23	1.20	0.75	0.45	7.02					
	period	48	1.30	0.80	0.50	7.02	6.91	1,458	1.72	2,500	27.6
M. S.	Control	24	1.10	0.20	0.90	8.7					
	period	29	1.05	0.30	0.75	8.7	9.0	1,100	1.72	1,890	
	Alcohol	30	0.70	0.20	0.50	4.34					
	period	55	0.56	0.25	0.31	4.34	5.42	1,750	1.72	3,000	58.0
E. R.	Control	33	1.75	1.05	0.70	6.0					
	period	38	1.75	1.15	0.60	6.0	6.0	924	1.76	1,620	
	Alcohol	19	1.15	0.80	0.35	5.4					
	period	39	1.10	0.70	0.45	5.4	5.44	1,430	1.76	2,510	55.0

* P—peripheral venous blood; H—hepatic venous blood; R—BSP removed, mg. per min.; Hct—hematocrit.

TABLE II

Combined data for infusions of saline and of saline-ethanol mixtures in five subjects *

Patient	Control period						Alcohol period							
	EHBF ml./min.		Glucose mg./100 ml.	Lactate mg./100 ml.	Pyruvate mg./100 ml.	Pepsinogen mcg. tyr./ml.	EHBF ml./min.		Glucose mg./100 ml.	Lactate mg./100 ml.	Pyruvate mg./100 ml.	Pepsinogen mcg. tyr./ml.	Alcohol mg./100 ml.	Acetaldehyde mg./100 ml.
L. S. 42 yrs. Gastric ulcer	1,770	P	100	17.9	2.0	—	2,380	P	94	26.0	2.8	—	28.0	0.13
		H	116	16.9	1.9	—		H	98	50.0	1.6	—	14.0	0.04
J. C. 48 yrs. Duodenal ulcer	895	P	104	6.6	1.8	0.61	1,340	P	83	10.4	1.9	0.59	81.5	0.42
		H	120	5.6	1.5	—		H	130	16.9	1.6	—	57.8	0.41
E. T. 31 yrs. Duodenal ulcer	1,960	P	88	17.4	2.0	0.67	2,500	P	88	30.2	1.4	0.69	47.5	0.24
		H	100	23.3	1.6	0.67		H	103	12.9	1.4	0.61	39.0	0.19
E. R. 39 yrs. Duodenal ulcer	1,620	P	98	17.2	—	0.64	2,510	P	89	21.3	2.0	0.68	25.5	0.32
		H	100	21.3	—	0.62		H	92	11.1	1.8	0.68	24.0	0.26
M. S.† 31 yrs. Duodenal ulcer	1,890	P	121	10.1	2.7	0.45	3,000	P	99	28.6	1.7	0.36	45.0	0.05
		H	129	14.8	3.3	0.22		H	127	29.8	1.3	0.35	38.0	0.04

* All calculations represent concentrations of substances per units of whole blood. P—peripheral venous blood; H—hepatic venous blood; EHBF—estimated hepatic blood flow; tyr.—tyrosine.

† Note: Patient M. S. had eaten a slice of toast.

infusions sufficient to maintain blood levels of this magnitude do not increase cardiac output, as measured by the dye dilution method (11); if any change occurs, cardiac output seems to be decreased by these levels of ethanol in the blood.

DISCUSSION

In the voluminous literature pertaining to the biochemical and physiological effects of ethanol on the mammalian body, only the recent study of Smythe, Heinemann, and Bradley (3) devotes attention to a comprehensive analysis of its action upon the hepatic blood flow. Their experiments were carried out on anesthetized dogs to whom ethanol was administered by gavage; the results may be summarized as showing no significant alteration in cardiac output, hepatic blood flow, or renal blood flow. The results of the present study are at variance with those reported by Bradley in that they show clearcut increases in the estimated hepatic blood flow produced by constant intravenous infusions of ethanol, at blood concentrations well below those necessary to produce symptoms of sedation or intoxication, or signs of peripheral vasodilation or of sweating. Not one of the subjects was able to recognize the taste, odor, or any other characteristics of ethanol during the course of the experiments. There were no changes in

TABLE III

Control study in one subject, utilizing saline infusion at three different rates, each rate being maintained for 30 minutes

Minutes after infusion began	Infusion rate		BSP mg. per 100 ml. serum			EHBF ml./min.	Glucose mg./100 ml. blood		Lactate mg./100 ml. blood		Pyruvate mg./100 ml. blood	
	Volume ml./min.	BSP mg./min.	P	H	P−H		P	H	P	H	P	H
24	20.0	11.0	2.5	0.55	1.95	960	99	113	16.4	8.7	1.88	1.88
54	9.0	4.95	1.4	0.65	0.75	1,050	98	112	10.5	13.1	1.68	1.52
84	8.0	4.4	1.35	0.55	0.80	940	93	108	14.9	8.9	1.98	1.66

TABLE IV

Cardiac indices in one subject during infusion of saline, saline + 5 per cent ethanol, and saline again, at a constant infusion rate of 8 ml. per minute

	Saline infusion No. 1		Ethanol infusion			Saline infusion No. 2
Minutes after infusion began	15 min.	18 min.	16 min.	30 min.	43 min.	12 min.
Cardiac index	5.2	5.4	4.7	5.10	4.7	5.3
Ethanol mg. per 100 ml. blood	—	—	(radial artery)—45 (antecubital vein)—42			— —

blood pressure. The fact that these increases in hepatic blood flow occurred in the absence of elevations of the peripheral BSP concentration must mean that the liver suffered no impairment of its ability to remove the dye during the alcohol period; there is no reason to suspect that extrahepatic removal of BSP was increased by the ethanol, although this possibility cannot be disproved. The many determinations of hepatic blood flow in this and in other laboratories do not support the possibility that infusions of 5 to 9 ml. of saline per minute *per se* increase hepatic blood flow, and there exists no evidence that ethanol itself causes an increase in cardiac output, of which the hepatic blood flow might partake proportionately. The simplest explanation would be that low peripheral blood levels of ethanol in unanesthetized man are associated with a decreased peripheral resistance in the splanchnic bed, giving rise to a significant increase in estimated hepatic blood flow.

The plasma pepsinogen levels were determined principally because in this laboratory it has been found that psychological stress rapidly elevates these values, in normal subjects as well as in those with peptic ulcers; these elevations are accompanied by changes in the output of gastric pepsin, but are reflected much more slowly in the urinary uropepsin excretion (12). Since these subjects all had peptic ulcer disease, it was felt that plasma pepsinogen values might be an excellent measure of the stress effect of the experiment. The fact that plasma pepsinogen failed to show significant increases during these studies, plus the relative stability of the blood glucose values, can be interpreted to mean that anxiety provoked by, or arising in the course of these experiments was of negligible significance.

It has been reported (13) that intravenous ethanol increases gastric secretion in normal subjects, although the increases described were not of great magnitude. In the studies reported here, there is no evidence that these blood levels of ethanol are associated with any elevation of the plasma pepsinogen concentrations over the control values. As has been pointed out by Mirsky, Futterman, Kaplan, and Broh-Kahn (8) the pepsinogen values of plasma are not necessarily influenced by secretagogues, but seem to reflect the interdigestive or continuous phase of gastric secretory activity.

Comparisons of ethanol concentrations in arterial and in peripheral venous blood in these studies and in the experiments of others (14) demonstrate a minimal peripheral disappearance of ethanol. All but one subject showed a significant hepatic uptake of ethanol; acetaldehyde was detected in both peripheral and hepatic venous blood, but the concentrations measured must represent only a small part of the acetaldehyde formed from these blood concentrations of ethanol. The present study provides no information as to the fate of the acetaldehyde formed; however, these data are in agreement with current theory as to conversion of ethanol via alcohol dehydrogenase to acetaldehyde and reduced DPN.

CONCLUSIONS

1. In each of five male subjects with peptic ulcer disease, the intravenous infusions of an ethanol-saline mixture maintaining blood ethanol levels at 25 to 81 mg. per 100 ml. caused a significant increase in estimated hepatic blood flow over values determined when saline solutions alone are administered at the same rate.

2. This increase in estimated hepatic blood flow resulting from ethanol cannot be explained on the basis of increased cardiac output, but seems best described as due to a lowering of the peripheral resistance of the splanchnic bed.

3. Metabolic data relating to the overall hepatic uptake of ethanol and production of acetaldehyde were consistent with present concepts of the intermediary metabolism of ethanol.

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THE ENZYMATIC DISSOLUTION OF EXPERIMENTAL ARTERIAL THROMBI IN THE DOG BY TRYPSIN, CHYMOTRYPSIN AND PLASMINOGEN ACTIVATORS [1, 2]

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A number of investigators have undertaken studies on the dissolution of intravascular thrombi and emboli by enzymes capable of effecting the dissolution of blood clots *in vitro* (1–4). Two types of approach have been used; the direct introduction of a proteolytic enzyme capable of digesting fibrin (2, 3), and activation of the naturally occurring proteolytic and fibrinolytic enzyme of mammalian plasma by the injection of a specific kinase (1). This latter approach is based on the observation that plasma contains a proenzyme termed plasminogen (5) or profibrinolysin (6), which, when activated, is converted to plasmin or fibrinolysin, a proteolytic enzyme with an affinity for fibrin (7). The action of plasmin is not limited to fibrin alone (8–11). The natural mechanisms for activation of plasminogen may be through blood (12) or tissue kinases (13, 14). The best known activator of human plasminogen is streptokinase, an extracellular hemolytic streptococcal product (8).

Recent reports have appeared on the successful liquefaction of intravascular clots in rabbit ear veins by the intravenous infusion of large amounts of streptokinase (1); the lysis of venous clots in rabbits and dogs by the intravenous administration of large amounts of trypsin (2); and the dissolution of venous thrombi in rabbits and dogs by injections of purified human plasmin (previously activated *in vitro* by streptokinase) (3).

It is the purpose of this report to describe the effects of the intravenous administration of trypsin, chymotrypsin and activators of plasminogen on the course of an experimentally induced femoral artery thrombosis in the dog. The results obtained suggest that the administration of chymotrypsin, or activation of the animals own plasminogen was associated with a significant increase in clot dissolution. Trypsin administration, however, was observed to have no significant effect on the experimental arterial thrombi.

MATERIAL AND METHODS

Production of experimental arterial thrombus. Femoral artery thromboses were produced in mongrel dogs weighing approximately 10 to 15 Kg. After surgical exposure of the artery a 1 to 2 cm. segment of the vessel was isolated between ligatures. Branches of the vessel were ligated and divided. The isolated segment was then distended with approximately 1 ml. of thromboplastin (Difco) solution.[3] The proximal ligature was removed and blood was allowed to reenter the previously isolated segment, where clotting slowly occurred. After a firm clot formed (usually 5 to 10 minutes), the distal ligature was removed. The artery was observed for another 5 minutes to verify the position of the clot. A black silk marker was loosely positioned about the obstructed vessel to aid in the subsequent identification of the segment. The wound was closed with Michel clips.

Enzyme preparations

Trypsin and chymotrypsin.[4] Preparations of trypsin and chymotrypsin were dissolved in Ringer's solution immediately prior to infusion. For trypsin, the amounts noted in the result section refer to mg. of tryptic activity since the preparations varied in their content of $MgSO_4$. For chymotrypsin, the amounts noted are the actual amounts infused. Infusions of these enzymes were given intravenously at a rate of 1 ml. per minute over a four-hour period using a constant infuson pump.

[1] Presented in part before the 26th Annual Meeting of the Central Society for Clinical Research, October 30th, 1953, Chicago, Illinois.

[2] This study was supported by grants from the Armour Laboratories, and from the Lederle Laboratories Division, American Cyanamid Co.

[3] Prepared as for prothrombin assay (15).

[4] Crystalline preparations of trypsin and chymotrypsin were obtained through the courtesy of Dr. A. H. Holland, Jr. of Armour Laboratories.

Plasminogen activators

Streptokinase or SK.[5] Units of SK are expressed in terms of the Christensen assay procedure (16). Preliminary *in vitro* studies revealed that activation of the plasminogen in dog plasma could be accomplished with approximately 200 units of this preparation of SK per ml. of dog plasma.[6] In the *in vivo* studies to be described, when SK alone was used as an activator, the material was dissolved in 30 ml. of saline and injected intravenously over a 5-minute period.

Human plasminogen preparation plus SK. In vitro studies revealed that the presence of small amounts of preparations of human plasminogen markedly facilitate the activation of animal plasminogen by SK (17). For *in vivo* studies a lyophilized human plasminogen preparation prepared from human plasma Fraction III[7] by the Christensen and Smith technique (18) was used. The preparation contained 4 per cent nitrogen and had a proteolytic assay, following SK activation, of 4.2 casein units per mg. nitrogen (11). Preliminary *in vitro* studies revealed that in the presence of 50 gamma of the above human plasminogen preparation, the plasminogen in 1 ml. of dog plasma was completely activated by only 20 units of SK. For use *in vivo*, the human plasminogen preparation was dissolved in 25 ml. of saline, mixed with 5 ml. of a saline solution containing the appropriate amount of SK, and the mixture injected intravenously over a 5-minute period. Although small amounts of human plasmin (SK activated) were injected with the activator mixture, it was demonstrated, *in vitro*, by adding equivalent amounts of spontaneously activated human plasmin[8] to dog plasma, that the amounts of human plasmin injected into these animals could only account for a very small part of the changes observed.

Other preliminary observations

A number of *in vitro* observations were made on the possible activation of dog plasminogen in plasma by trypsin and chymotrypsin, and on the inhibition of trypsin by plasma. Although trypsin appears to be an activator of partially purified plasminogen (19), studies *in vitro* with trypsin and chymotrypsin failed to reveal any activation of the plasminogen in undilute plasma. The levels of free tryptic activity observed after the addition of trypsin to undilute plasma, was found to represent about 1 to 3 per cent of the enzyme added (up to 2 mg. per ml. plasma), suggesting that the trypsin in undilute plasma may combine reversibly with the plasma inhibitors (20). The type of inhibition observed in undilute plasma is in contrast to the type of inhibition described with dilute plasma or purified systems where specific plasma inhibitors which combine with trypsin in stoichiometric fashion may be demonstrated (21, 22).

Experimental design. For purpose of comparing the effects of each of the preparations used, a standard experimental procedure was followed. Twenty-four hours after the production of the experimental thrombus, treatment with one of the agents was begun, in doses referred to in the results section. Single treatments consisting of 4-hour infusions of trypsin or of chymotrypsin, or an intravenous injection of SK, or SK plus human plasminogen were given on two successive days. Four days following the initiation of therapy the previously thrombosed vessel was exposed, studied and biopsied.

Animals receiving trypsin and chymotrypsin infusions were maintained under pentothal anaesthesia during the infusion period. When injections of SK plus plasminogen, or SK alone were given, no anaesthesia was employed.

Evaluation of results of clot dissolution. Results were classified into successes or failures depending upon whether an effective circulation was restored in the previously thrombosed vessel as judged by gross inspection, presence of a pulse, back bleeding when the artery was divided proximal to the previously thrombosed area, and by gross and histological examination of the biopsied specimen. Failures included all instances in which the lumen remained obstructed by a thrombus even though the thrombi may have shown evidence of partial lysis presumably from the enzyme treatment. Successes included all instances in which adequate circulation was restored even though histological examination occasionally revealed small nonobstructive fragments of thrombus still adherent to the endothelium. No attempt was made to grade the clots for partial lysis since this would require absolutely uniform clots.

Biochemical studies. Biochemical studies, carried out on venous blood removed *before, during,* and *after* the treatment with each agent included the Lee White clotting time, and the subsequent lysis time of the clotted specimen; fibrinogen (23); prothrombin (15);[9] proteolytic activity (24); plasminogen (16); and the presence of an active fibrin lysing system in the dogs plasma (25). On a number of instances the presence of circulating plasmin or trypsin was tested for by the hydrolysis of p-toluene sulfonyl l-arginine methyl ester (26, 27), and the presence of circulating chymotrypsin tested for by the hydrolysis of acetyl l-tyrosine ethyl ester (27).

[5] A commercial preparation (Varidase) Lot No. 7-1089-297A, containing only small amounts of contaminating inhibitor, was obtained through the courtesy of Dr. J. M. Ruegseggar of Lederle Laboratories.

[6] We refer here to that amount of SK which when added to 1 ml. of dog plasma, prior to clotting the mixture with thrombin, produced clot lysis within 30 minutes. The test system consisted of 1.0 ml. dog plasma plus 0.1 ml. SK solution plus 0.1 ml. Bovine Thrombin solution containing 1 NIH unit.

[7] Obtained from the American Red Cross, and through the courtesy of Dr. T. D. Gerlough of E. R. Squibb & Sons.

[8] Obtained through the courtesy of Dr. D. M. Surgenor, Harvard Univ. Lab. of Physical Chemistry (Fraction III_3).

[9] Modified by the addition of 0.1 ml. of 2.5 per cent clottable fibrinogen in saline to test system.

RESULTS

Natural course of the experimental thrombus

Femoral artery thromboses were produced in 29 animals which received no form of therapy. Upon examination 5 days later, 26 of the clots were still *in situ* and exhibited the earliest stages of organization. Three (10 per cent) of the clots had spontaneously disappeared. In several other animals surgical exposure of the vessels was not carried out until two weeks after the formation of the thrombus. At this time the clots were still present and exhibited evidence of advancing organization.

Trypsin treated animals

In 22 instances trypsin was infused at a dose of 16 to 33 mg. per Kg. Only two of these clots dissolved (9 per cent of the total).

Typically, a trypsin infusion caused a prompt rise in circulating proteolytic activity, which per-

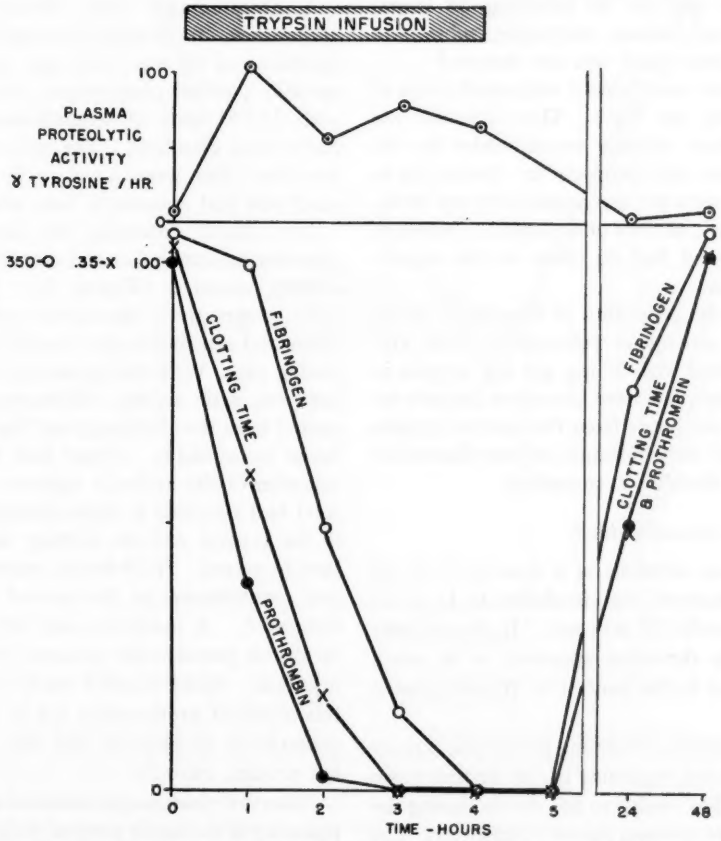

FIG. 1. THE EFFECTS OF A FOUR-HOUR INTRAVENOUS INFUSION OF 300 MG. OF TRYPTIC ACTIVITY INTO A 12 KG. DOG

The abscissa represents time in hours. The ordinate is divided into two parts; at the top is shown the concentration of plasma proteolytic activity (gamma acid soluble tyrosine released per hour); in the lower section is shown the reciprocal of the whole blood clotting time (a measure of the clottability of the blood) ×—×, the prothrombin concentration (per cent control) ●—●, and fibrinogen concentration (mg. per cent) ○—○. The ordinate is designed so that at 100 per cent prothrombin, the reciprocal of the clotting time is 0.35, and the fibrinogen concentration is 350 mg. per cent.

sisted during the period of infusion and returned to the control value by the next day (Figure 1). Prothrombin rapidly fell to zero and the blood became incoagulable. Fibrinogen also disappeared from the circulation but at a somewhat slower rate. Although these latter effects were of a transient nature, 48 hours were required for a complete return to the control value.

Activation of the animals plasminogen *in vivo* could not be demonstrated. Plasminogen levels were found to remain constant throughout the infusion periods and for the following 24 hours. When the blood became coagulable, subsequent lysis of the clotted blood was not observed

Three animals were infused with small doses of trypsin (0.4 mg. per Kg.). These amounts are higher than those recently recommended by Innerfield, Angrist, and Schwarz for clinical use in man (28) but were not accompanied by any measurable proteolytic activity or changes in fibrinogen concentration, and had no effect on the experimental thrombi.

To exclude the possibility of dissolution of the clot and then subsequent reformation, three animals were infused with 25 mg. per Kg. trypsin in the usual manner, but were also given heparin injections every six hours from the onset of trypsin therapy. In all three instances a firm obstructing thrombus was found at examination.

Chymotrypsin treated animals

Chymotrypsin infusions at a dose of 12 to 30 mg. per Kg. restored the circulation in 13 of 25 thrombosed vessels (55 per cent). In the majority of failures, the thrombus appeared to be much softer than that in the control or trypsin treated animals.

Characteristically, circulating proteolytic activity rapidly rose at the beginning of the chymotrypsin infusion, and then tended to fall slowly during the remainder of the infusion period (Figure 2). The prothrombin concentration was not affected. The fibrinogen concentration fell to about 50 per cent of the control value and was maintained at about that level during the infusion. The clottability of the blood was similarly affected. Restoration to the control values was complete within 24 hours.

No evidence of plasminogen activation could be obtained either by plasminogen analyses, or by observations on the lysis of clotted blood removed during or after the infusion period.

The dissolution of the experimental thrombus with chymotrypsin appeared to bear a relation to dosage. In 10 animals treated with chymotrypsin at a dose level of 3 to 10 mg. per Kg., less profound changes in the fibrinogen concentration and coagulation times were noted, and only three of the clots dissolved.

Animals treated with activators of plasminogen

A. *Streptokinase plus human plasminogen preparation.* In 28 animals treated by intravenous injections of 60 mg. (2.4 mg. nitrogen) of the partially purified plasminogen preparation mixed with 25,000 units of streptokinase, 14 (50 per cent) clots dissolved. The majority of the undissolved clots were noted to be very soft and small and had apparently been altered.

Immediately following the injection of the activator mixture, a burst of circulating proteolytic activity occurred (Figure 3). Proteolytic activity progressively decreased over the next few hours and returned to the control level by the following day. With the appearance of the circulating proteolytic activity, fibrinogen rapidly disappeared from the circulation and the blood was rendered incoagulable. About four hours after the injection of the activator mixture, the fibrinogen level had returned to approximately 50 per cent of the control and the clotting time was almost back to normal. At 24 hours, return of the fibrinogen concentration to the control level had been completed. A moderate and very transient fall in plasma prothrombin occurred shortly after the injection. More detailed study has shown that this apparent prothrombin fall is due to a disappearance of Ac globulin, and that true prothrombin remains intact.[10]

Assays of plasminogen revealed a striking disappearance of the major portion of this proenzyme at the time of the appearance of the circulating proteolytic activity, and the slow reappearance of this plasma constituent to control levels by the next day. The plasma of animals treated with the activator mixture were able to dissolve standard

[10] We are indebted to Dr. Helen I. Glueck for these studies. That Ac globulin is attacked by plasmin has been previously reported (10).

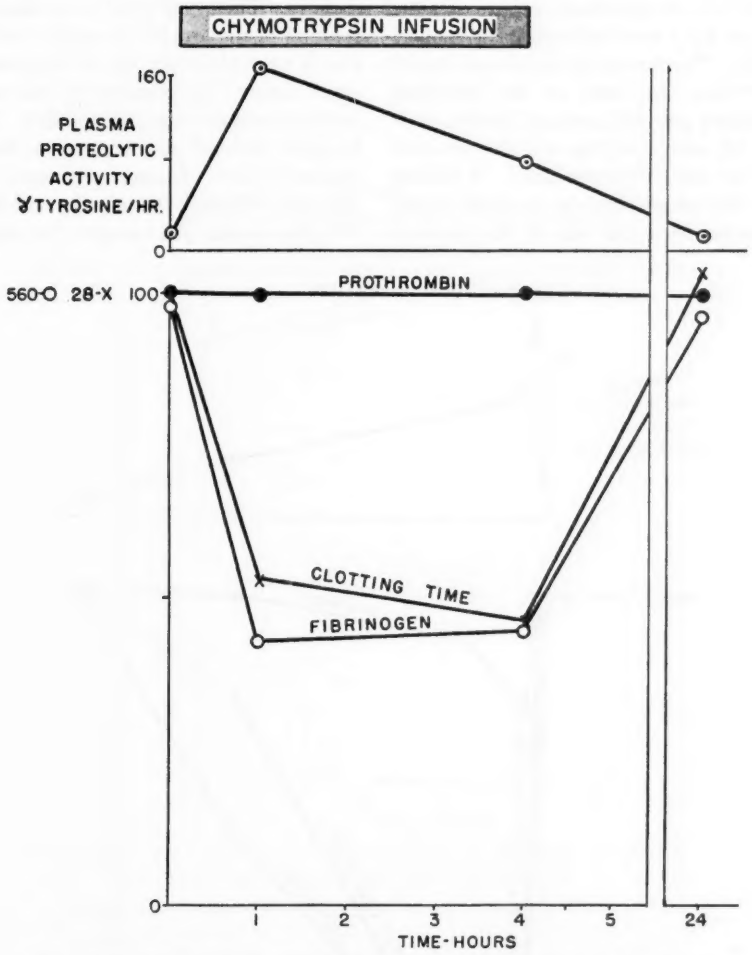

FIG. 2. THE EFFECTS OF A FOUR-HOUR INTRAVENOUS INFUSION OF 200 MG. OF CHYMOTRYPSIN INTO A 10 KG. DOG.

The abscissa represents time in hours. The ordinate plots plasma proteolytic activity (gamma acid soluble tyrosine released per hour) in the top section of the figure; and the prothrombin concentration (per cent control) ●—●, the reciprocal of the whole blood clotting time ×—×, and the fibrinogen concentration (mg. per cent) ○—○, in the bottom section of the figure. The ordinate is designed so that at 100 per cent prothrombin, the reciprocal of the clotting time is 0.28, and the fibrinogen concentration is 560 mg. per cent.

bovine fibrinogen-bovine thrombin clots within 30 minutes for periods of one to two hours following the injection. Observations of this type could not be demonstrated in the trypsin and chymotrypsin treated animals.

No attempts were made to vary the amounts of each of the components of the activating mixture. In a small series of animals, either 60 mg. of the partially purified plasminogen mixture, or 25,000 units of SK were injected alone, without evidence of activation of plasminogen or of clot dissolution.

B. *Streptokinase alone.* Two hundred and fifty

thousand units of streptokinase (approximately 20,000 units per Kg.) were injected intravenously into 26 animals. This dose of streptokinase was 10 times greater than that used in the activating mixture containing streptokinase and human plasminogen. In 13, or half of the animals, the clots were gone at the time of examination. A finding of interest in this group was the frequent occurrence of a hematoma at the site of the previous surgery. The bleeding bore no relation to the dissolution of the arterial thrombus and was suggestive of lysis of the thrombi in the small vessels incised during the exposure of the artery for the production of the experimental clot. In spite of the frequent presence of a hematoma, the wound was unusually clean. Clean experimental wounds were also seen following the injections of the mixture of SK plus human plasminogen, but not in the con-

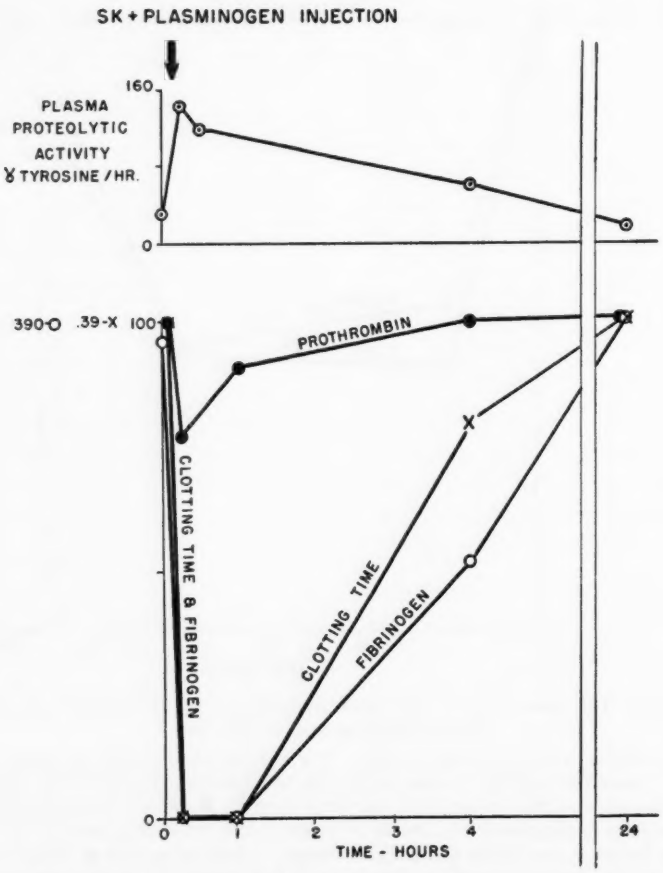

FIG. 3. THE EFFECTS OF A SINGLE INTRAVENOUS INJECTION OF A MIXTURE OF 25,000 UNITS OF STREPTOKINASE PLUS 60 MG. OF A PARTIALLY PURIFIED HUMAN PLASMINOGEN PREPARATION IN A 13 KG. DOG

The abscissa represents time in hours. The injection was given immediately following the removal of the control blood sample. The ordinate plots plasma proteolytic activity (gamma acid soluble tyrosine released per hour) in the top section of the figure; and the prothrombin concentration (per cent control) ●—●, the reciprocal of the whole blood clotting time ×—×, and the fibrinogen concentration (mg. per cent) ○—○, in the bottom section of the figure.

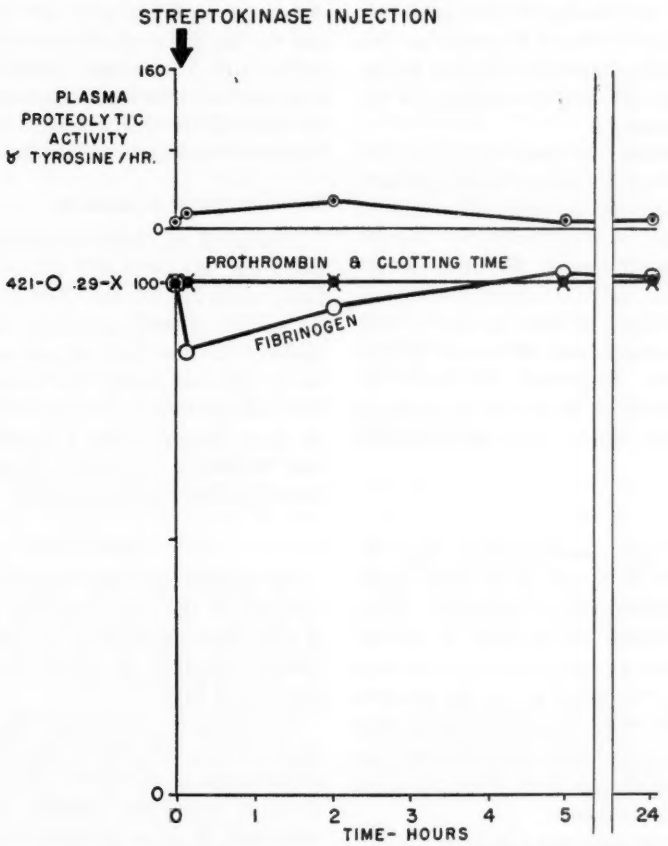

FIG. 4. THE EFFECTS OF A SINGLE INTRAVENOUS INJECTION OF 250,000 UNITS OF STREPTOKINASE INTO A 12 KG. DOG

The abscissa represents time in hours. The injection was given immediately following the removal of the control blood sample. The ordinate plots plasma proteolytic activity (gamma acid soluble tyrosine released per hour) in the top section of the figure; and the prothrombin concentration (per cent control) ●—●, the reciprocal of the whole blood clotting time ×—×, and the fibrinogen concentration (mg. per cent) ○—○, in the bottom section of the figure.

trol, trypsin or chymotrypsin treated animals. Following the injection of SK, there was typically a very slight increase in circulating proteolytic activity (Figure 4). This increase was maintained for at least two hours but had completely disappeared by the fifth hour. The plasma prothrombin concentration, and the clotting time of the whole blood remained unaffected by the injection. A relatively minor, but significant, fall (15 per cent) in the fibrinogen concentration of short duration occurred with restoration to normal by the fifth hour.

Although only a slight increase in proteolytic activity, and no alteration in clotting time, was observed, the specimen of blood removed ten minutes following the SK injection promptly lysed within 30 minutes of coagulation. No lysis in 48 hours was noted with any of the other specimens removed prior to, or at later intervals, following the injection of SK. Plasminogen assays revealed a

25 per cent fall in this proenzyme titer in the 10-minute specimen, and a return to normal at two hours. These results suggested a limited activation of plasminogen following the injection of SK at the dose level employed.

Table I summarizes the observations on clot dissolution for each of the various agents utilized.

Trypsin apparently had no effect above that of the spontaneous rate of clot dissolution. Significant increase in the per cent of clots dissolved appears to have been produced by infusions of chymotrypsin and by injections of large amounts of SK, or by injections of an activating mixture of SK plus human plasminogen. In general, the results obtained on clot dissolution at the doses employed with the latter three agents, were approximately the same.

Toxicity

No significant toxic manifestations were observed with any of the agents at the dose levels, rate, and route of administration employed. When the blood was rendered incoagulable by enzyme treatment, a tendency to ooze blood from the sites of needle puncture was noted during the duration of the incoagulable state. Animals injected with the mixture of SK plus human plasminogen were up and about even though their blood remained incoagulable for several hours.

The question of embolization following clot dissolution could not be adequately studied since the clots were on the arterial side of the circulation, and the dog has an extensive collateral circulation in his lower extremities. Although emboli were searched for in the distal segment and branches of the femoral artery, none were found. However, this examination was limited in extent.

TABLE I

Summary of results on the dissolution of intravascular clots in vivo by enzymatic agents

Agent and dose	Number of clots	Number of clots dissolved	Number of clots remaining	Percentage of clots dissolved
Control	29	3	26	10
Trypsin				
0.4 mg./Kg.	3	0	3	—
16–33 mg./Kg.	22*	2	20	9
Chymotrypsin				
3–10 mg./Kg.	10	3	7	30
12–30 mg./Kg.	25	13	12	55
SK (25,000 units) plus human plasminogen (60 mg.)	28	14	14	50
Streptokinase (250,000 units)	26	13	13	50

* Does not include three instances where heparin therapy was given in conjunction with trypsin therapy, with failure of the clots to dissolve.

Rapidity of clot dissolution

Because of the design of the experiment, quantitative data on the speed of clot dissolution with these various agents was not obtained. In each of the groups treated, occasional observations were made of the previously thrombosed artery within six to eight hours after the therapeutic agent had been administered. In several instances, in each of those groups where a significant increase in clot dissolution occurred, disappearance of the thrombus had already occurred.

DISCUSSION

An attempt has been made in this report to evaluate, in the dog, a number of agents which, *in vitro,* have the capacity for either directly or indirectly effecting the rapid dissolution of fibrin and clotted blood.

Crystalline trypsin did not appear to be an ideal agent for clot dissolution since it readily converts prothrombin to thrombin (29). When added to blood in insufficient quantity to destroy all the fibrinogen, it is an excellent blood clotter (29). In addition, it is extensively inhibited by plasma (21, 22), and in partially purified systems appears to attack fibrinogen more readily than fibrin (30). The results observed in the dogs were consistent with this view. Prothrombin was destroyed at a faster rate than fibrinogen and no significant clot lysis or activation of plasminogen was observed. Although most of our biochemical findings agreed with those previously reported by Innerfield, Schwarz and Angrist (2), their observations on clot dissolution were not confirmed. Perhaps the fact that the thrombi in the present experiments were arterial rather than venous may account for some of the differences noted. When small amounts of trypsin were used, no evidence of any increased circulating proteolytic activity was observed.

Chymotrypsin, the other major proteolytic enzyme of the pancreas, rapidly splits fibrinogen and

fibrin. It does not destroy prothrombin or clot blood, and would appear to be more suitable than trypsin for intravascular clot dissolution. Chymotrypsin is extensively inhibited by plasma (31) but comparatively little is known about this inhibition. In partially purified systems chymotrypsin appears to act on fibrinogen more readily than fibrin. The results obtained *in vivo* indicated that the major portion of the infused enzyme was rapidly inhibited since only moderate fibrinogenolysis was observed. Activation of plasminogen could not be demonstrated. Surprisingly, however, a large percentage (55 per cent) of the thrombi were dissolved. It has been suggested that chymotrypsin may facilitate clot dissolution by lowering the antifibrinolytic activity of blood (32).

On theoretical grounds plasmin would appear to be the enzyme of choice for clot dissolution. It is naturally occurring, is inhibited less than the pancreatic enzymes, does not clot blood, has restricted activity in plasma, and in purified systems appears to split fibrin as readily, if not more readily than fibrinogen. The *in vitro* observations of the activation of dog plasminogen by SK in the presence of partially purified human plasminogen (17), allowed us to activate dog plasminogen *in vivo* with small doses of SK. Although this method of activation may be applied to the dog, rabbit, and cow, and perhaps other animals as well, it does not facilitate the activition of human plasminogen (17). The per cent of clots which dissolved following the injection of the activation mixture are not as high as reported by Cliffton, Grossi, and Cannamela (3), in a much smaller series of dogs. Their studies were confined almost entirely to venous thrombi, and earlier treatment of the experimental thrombus was frequently instituted.

The results obtained with injections of streptokinase alone as an activator, contrast quite sharply with the SK plus human plasminogen injections in so far as fibrinogenolysis and circulating proteolytic activity are concerned, yet are similar in their effect on fibrin and the dissolution of arterial clots. This special fibrinolytic activity seen after *in vivo* injections of streptokinase alone has been previously noted by Johnson and Tillett (1). In purified *in vitro* systems, plasmin appears to attack fibrinogen and fibrin at a similar rate (8). The *in vitro* studies of Ratnoff (33) would suggest that the explanation for this disparity may be an accelerated activation of plasminogen on the fibrin clot. Recent *in vitro* studies, suggest that, in the presence of SK, fibrin extracts plasmin from a reversibly inhibited system existing in plasma. Such a mechanism would also explain the differences noted between the native plasma as compared to purified systems. In contrast to the observations with SK alone, the presence of the mixture of SK plus human factor probably removed the plasma inhibition and allowed for a more complete activation of the plasminogen in the dog plasma, resulting in extensive fibrinogenolysis and other evidences of proteolysis. The special fibrinolytic activity observed after the injections of streptokinase alone should serve as a stimulus for further purification of this activator.

In each of the groups where significant clot dissolution was observed, approximately half the clots still remain undissolved. In the majority of these undissolved clots, evidence of partial dissolution was present. Modifications in treatment which may, or may not, have produced a higher percentage of clot dissolution were not investigated.

SUMMARY

1. Experimental femoral artery thrombi have been produced in dogs, and a study made of their dissolution by the intravenous administration of trypsin, chymotrypsin, and two types of plasminogen activators.

2. Trypsin in doses sufficient to produce a significant increase in circulating proteolytic activity and to destroy all the circulating prothrombin and fibrinogen, did not enhance the dissolution of the experimental arterial thrombus.

3. Chymotrypsin, at a dose range similar to that of trypsin, produced similar increases in circulating proteolytic activity but only a moderate fibrinogenolysis, and no effect on circulating prothrombin, but was associated with a dissolution of 55 per cent of the clots. No activation of plasminogen was demonstrable, and the mechanism of the action on the thrombi is not apparent.

4. Following the injection of a mixture of moderately small amounts of streptokinase plus a partially purified human plasminogen preparation, rapid activation *in vivo* of dog plasminogen resulted. The activation of dog plasminogen was accompanied by a significant increase in circulating proteolytic activity and rapid disappearance of

the plasma fibrinogen with a resultant incoagulable state. The activation of plasminogen in the plasma by this method was associated with a dissolution of 50 per cent of the clots.

5. Injections of streptokinase alone promoted clot dissolution (50 per cent) and the rapid liquefaction of normally clotting blood specimens, but produced only moderate changes in the plasma fibrinogen and plasminogen levels. Minor increases in circulating proteolytic activity were demonstrable. The data are consistent with the view that, *in vivo,* injections of streptokinase alone are associated with greater fibrinolytic, as compared to fibrinogenolytic, activity.

6. No toxicity was noted with any of the agents studied with the dose, rate and method of administration employed.

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PHAGOCYTOSIS BY HUMAN LEUCOCYTES. I. EFFECT OF FIBRIN ON PHAGOCYTOSIS OF STAPHYLOCOCCI AND OF ENCAPSULATED PNEUMOCOCCI BY NORMAL HUMAN LEUCOCYTES[1]

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Resistance to phagocytosis by pathogenic microorganisms may be due to a variety of mechanisms depending on the species. For example, bacteria may produce substances toxic for leucocytes such as the "leucocidins" produced by staphylococci and hemolytic streptococci. Another mechanism suggested by Hale and Smith (1) may operate in the case of pathogenic staphylococci; these bacteria produce an enzyme, coagulase, which acting in concert with a serum factor catalyzes the conversion of fibrinogen to fibrin. According to Hale and Smith, the fibrin meshwork so formed, impedes the activity of the leucocytes, and furthermore, fibrin, intimately deposited about the staphylococci, in some way interferes with their ingestion.

Rogers and Tompsett (2), using human leucocytes, confirmed the observation that coagulase-producing staphylococci are less well ingested than coagulase negative strains, but believe that the observed difference was not of sufficient magnitude to be a factor of major importance in explaining the difference in pathogenicity between the two species.

The production of capsules by certain bacterial species can be correlated with pathogenicity. Specific anticapsular serum renders the encapsulated organism relatively defenseless to leucocyte activity. Wood, Smith, and Watson (3, 4) have shown, however, that encapsulated pathogens may be ingested in the absence of specific antibody both *in vitro* and *in vivo* provided the leucocytes can push the organisms against a nonyielding surface. A fibrin meshwork such as occurs in blood clots or inflammatory exudates offers an excellent matrix for phagocytosis in the absence of antibody (5). The validity of "surface phagocytosis" is unquestioned, but its quantitative significance *in vivo* has not been defined.

The observed difference in the effect of fibrin on the ingestion of coagulase positive staphylococci and encapsulated pneumococci suggests that enzymatic therapy designed to reduce the amount of fibrin in inflammatory lesions (6, 7) may increase or diminish natural phagocytosis depending on the microorganisms involved.

An attempt has been made in the present study to confirm the observations of Hale and Smith (1) on the influence of coagulase production by staphylococci on phagocytosis and to determine the effect of streptokinase on this process. In addition, surface phagocytosis of type II encapsulated pneumococci has been studied quantitatively and the effect of streptokinase on it has been evaluated.

METHODS

The influence of coagulase on the ingestion of staphylococci by human leucocytes was determined by (a) comparing the ingestion of coagulase positive with the ingestion of coagulase negative staphylococci under identical conditions, and (b) by comparing the ingestion of coagulase positive strains in coagulable and noncoagulable media.

Roller tube technique

One-tenth milliliter of a standardized suspension of organisms was added to 1.0 ml. human blood (either heparinized, streptokinase-treated, defibrinated, or defibrinated plus streptokinase). The mixtures were placed in glass tubes 12 × 0.8 cm. in size, sealed and rotated at 8 R.P.M. in an incubator at 37° C. for one hour. Smears were made and phagocytosis recorded as the average number of organisms ingested per polymorphonuclear leucocyte; neutrophils were counted regardless of whether ingestion had occurred. Four coagulase positive staphylococcal strains (I, II, III, and IV) and one coagulase negative strain were used. Staphylococci were grown in

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fresh beef heart infusion broth containing 1 per cent neopeptone; overnight cultures were used which were centrifuged, and suspensions standardized turbidimetrically so as to contain approximately 1.5×10^9 organisms per ml. Suspensions were tested for coagulase production in each experiment, using the tube test.

Blood samples were handled as follows: 18 ml. was drawn into a dry syringe and rendered noncoagulable by the addition of approximately 2 mg. heparin. A second specimen was drawn into a dry syringe and rendered noncoagulable by two methods: (a) the addition of streptokinase which promotes the breakdown of fibrin and fibrinogen (blood plus streptokinase), and (b) by defibrination with the use of glass beads (defibrinated blood). In the former method, the quantity of streptokinase (Varidase, Lederle) used was arbitrarily chosen as the least amount which would cause lysis in less than 2 minutes at 37° C. when incorporated in a plasma clot. The amount used to defibrinate blood was approximately 800 units per ml. of blood, and varied to some extent with different batches of enzyme. The designation, blood plus streptokinase, used in this paper refers to blood which had clotted but in which fibrinolysis had occurred subsequently due to enzyme activity. In the second method of defibrinating blood, many leucocytes are caught in the fibrin strands about the beads; therefore, it is necessary to add viable leucocytes to the defibrinated blood. This was accomplished by adding the washed red and white cells from a known volume of heparinized blood to the serum from an equal volume of defibrinated blood from the same donor. Cells were washed twice in Tyrode's solution and were centrifuged at 4° C. A control specimen of blood used in these experiments was labelled defibrinated blood plus streptokinase and consisted of defibrinated blood to which approximately 800 units of streptokinase had been added. The dilution of blood by streptokinase was controlled by the addition of an equal volume of 0.9 per cent saline to the other preparations (14 per cent of total volume of blood). The number of leucocytes per cubic millimeter was determined for each preparation of blood. Blood used was obtained from 17 healthy human donors.

Technique for surface phagocytosis

Surface phagocytosis was studied quantitatively in the following manner: 1.5 ml. of blood (either heparinized, streptokinase-treated, or defibrinated) was mixed with 0.2 ml. of a standardized suspension of organisms (1.5×10^9 per ml.) in a 15-centimeter Petri dish to which 0.1 ml. of thrombin was added. A clot formed in only the heparinized blood. The mixture was then incubated at 37° C. for one hour at which time 0.5 ml. of streptokinase (1,250 units) was layered on the preparation. After further incubation for one hour, smears were made from the liquefied mixture. Phagocytosis was recorded as the average number of organisms ingested per neutrophilic polymorphonuclear leucocyte. In this way a comparison was obtained of the ingestion of four different bacterial strains under the same conditions: coagulase positive staphylococcus, coagulase negative staphylococcus, encapsulated type II pneumococcus (strain D39S), where virulence was maintained by mouse passage, and a rough mutant of the latter (strain 36A). Ingestion of type II pneumococcus was also measured in the presence of type II antipneumococcal rabbit serum. Control mixtures contained normal rabbit serum in place of antipneumococcal serum. Four-hour 0.1 per cent glucose beef heart infusion broth cultures of pneumococci were used, centrifuged and standardized turbidimetrically to contain approximately 1.5×10^9 organisms per ml. Staphylococci were prepared as described under the roller tube technique. Phagocytosis of each bacterial strain was determined in three different blood preparations: heparinized blood, blood defibrinated by streptokinase (800 units per ml.), and blood defibrinated by shaking with glass beads as described above. The number of leucocytes per cubic millimeter was counted in each blood preparation so that the ratio of organisms to leucocytes in each mixture was known. Blood from a series of 20 healthy human donors was used in tests carried out in Petri dishes.

RESULTS

Roller tube technique

Table I records mean ingestion of (C+) and (C−) staphylococci by neutrophils in four different blood preparations: blood plus heparin, blood plus streptokinase, defibrinated blood, and defibrinated blood plus streptokinase. Mean ingestion of all coagulase positive strains (I, II, III, and IV) has been recorded in each preparation because this was not significantly different with individual strains.

In comparing neutrophil ingestion, it may be important to keep the ratio of bacteria to phagocytes constant (8, 9). In the present experiments the bacterial suspensions used were always adjusted to the same turbidity and the neutrophil count per cubic millimeter determined for each blood preparation. The over-all ratio averaged 1 neutrophil to 50 bacteria. Since bacteria were always present in considerable excess, the differences in ratio of bacteria to neutrophils in different experiments is probably not an important factor. In any event, under the conditions of our experiments, the degree of phagocytosis appeared to be independent of the neutrophil count. A similar observation was recorded by another group of investigators (10).

Ingestion of (C−) staphylococci. There was no significant difference in the ingestion of (C−) staphylococci by neutrophils in heparinized blood, streptokinase-treated blood, blood defibrinated

with glass beads, or in the latter containing streptokinase. Therefore, it may be concluded that in these experiments, heparin and streptokinase did not affect phagocytosis; this agrees with the findings of others (11–13). The data also show that the removal of fibrinogen with glass beads does not influence ingestion.

Ingestion of (C+) staphylococci. There was a significant reduction in the mean ingestion of (C+) staphylococci by neutrophils in heparinized blood compared with their mean ingestion by neutrophils in all the other blood preparations. However, significantly fewer (C+) staphylococci were ingested by leucocytes in streptokinase-treated blood compared with leucocytes in blood defibrinated by glass beads. The addition of streptokinase to the latter did not significantly alter the phagocytic index.

These data showed that coagulase-producing staphylococci were less readily ingested in the presence of fibrin, which agrees with the findings of Hale and Smith (1) and Rogers and Tompsett (2). But under the conditions of the present experiments considerably more (C+) staphylococci were ingested in the presence of fibrin than reported by the above authors.

The diminished ingestion of (C+) staphylococci in streptokinase-treated blood was an unexpected finding. This was probably not due to a direct effect of streptokinase on leucocytes because a comparable amount of the enzyme did not affect the ingestion of either (C−) or (C+) staphylococci in the absence of fibrinogen. It might have been due to incomplete removal of fibrinogen by streptokinase, although sufficient enzyme was added to lyse completely the clot which formed spontaneously after drawing the blood; moreover, there was no gross or microscopic evidence of fibrin in the streptokinase-treated blood after incubation for one hour with (C+) staphylococci. However, special fibrin stains were not used in these studies.

Ingestion of (C+) staphylococci compared with ingestion of (C−) staphylococci. The mean ingestion of (C−) staphylococci was greater than that of (C+) staphylococci in heparinized blood. This difference was significant. In contrast phagocytosis of (C+) staphylococci was greater than that of (C−) staphylococci both in blood defibrinated by glass beads and in defibrinated blood to which streptokinase had been added. There was no significant difference in the mean ingestion of the two species of staphylococci in the streptokinase-treated blood. It is possible that the latter finding was due to incomplete removal of fibrinogen which resulted in decreased ingestion of (C+) strains as discussed above.

Petri dish technique

The Petri dish technique was devised for two reasons; first, to attempt to record quantitatively the phagocytosis of (C+) staphylococci exposed

TABLE I

Ingestion of staphylococci by neutrophils in blood containing heparin or streptokinase, or in blood defibrinated with glass beads
(Roller tube technique—17 subjects)

Blood preparation	Average neutrophil count per cu. mm.	Number neutrophils counted	Micro-organism*	Mean ingestion per neutrophil	Standard error
Blood plus heparin	3,770	3,400	C+	15.7	±1.0
		800‡	C−	20.4	±1.8
Blood plus streptokinase	3,500	3,400	C+	23.0	±2.8
		850	C−	23.7	±2.6
Defibrinated blood†	3,540	3,400	C+	32.7	±1.6
		850	C−	22.0	±1.7
Defibrinated blood† plus streptokinase	3,540	3,400	C+	33.5	±1.6
		850	C−	23.9	±1.9

* C+ = coagulase positive strains I, II, III, and IV.
C− = coagulase negative.
† Defibrinated with glass beads.
‡ 16 subjects.

only to neutrophils in a fibrin clot. This was accomplished by adding thrombin to blood as soon as microorganisms and neutrophils were mixed. Hale and Smith (1) pointed out that the ingestion of (C+) staphylococci in plasma probably occurs before fibrin has been deposited as a result of coagulase activity. However, in warm stage preparations leucocytes may be seen to move in a fibrin clot, so it seemed important to attempt to measure phagocytosis when fibrin was present at the initial exposure of microorganisms to leucocytes. A second reason for using the Petri dish technique was to compare the ingestion of type II encapsulated pneumococci in the presence or absence of a fibrin clot. The effect of type specific antibody under the same conditions could then be studied.

The mixtures were not agitated constantly during incubation; but because the average ratio of bacteria to neutrophils was approximately 50:1, it is reasonable to assume that there was adequate contact between them.

Table II records data on the phagocytosis of staphylococci and pneumococci in three different blood preparations: blood plus heparin, blood plus streptokinase, and blood defibrinated with glass beads.

Ingestion of (C−) staphylococci. There was no significant difference in the ingestion of (C−) staphylococci by neutrophils in any of the blood preparations, showing that in these experiments, thrombin, heparin, streptokinase, or defibrination with glass beads did not affect phagocytosis.

Ingestion of (C+) staphylococci. The mean ingestion of (C+) staphylococci by neutrophils was greatest in defibrinated blood, somewhat smaller in heparinized blood, and least in streptokinase-treated blood. These differences are statistically significant. There is no obvious explanation for the relatively low mean ingestion in streptokinase-treated blood unless it was the presence of an additional 1,200 units of streptokinase in this preparation. But, as reported above, under identical conditions no significant difference was found in the ingestion of (C−) staphylococci. The mean ingestion of (C+) staphylococci by neutrophils in heparinized blood was higher in Petri dishes than in roller tubes. This was probably due to the addition of streptokinase to the former at the end of one hour's incubation; fibrinolysis

TABLE II

Ingestion of microorganisms in blood containing heparin, or streptokinase, or in blood defibrinated with glass beads (Number neutrophils counted per microorganism—1,000, Petri dish technique, 20 subjects)

Blood preparation*	Average neutrophil count per cu. mm.	Microorganism	Clot formed after addition of thrombin	Mean ingestion per neutrophil	Standard error
Blood plus heparin	4,200	Staphylococcus (C+)†	+	25.3	±1.1
		Staphylococcus (C−)	+	15.4	±0.9
		Rough pneumococcus	+	21.7	±1.3
		Type II pneumococcus	+	7.8	±0.4
		Type II pneumococcus plus antibody	+	11.2	±1.1
Blood plus streptokinase (1,200 units)	3,800	Staphylococcus (C+)†	−	19.5	±1.9
		Staphylococcus (C−)	−	11.6	±1.9
		Rough pneumococcus	−	21.8	±2.1
		Type II pneumococcus	−	0.3	±0.2
		Type II pneumococcus plus antibody	−	12.0	±2.3
Defibrinated‡ blood	4,100	Staphylococcus (C+)†	−	33.6	±2.5
		Staphylococcus (C−)	−	14.8	±1.3
		Rough pneumococcus	−	28.3	±2.7
		Type II pneumococcus	−	0.7	±0.2
		Type II pneumococcus plus antibody	−	13.0	±1.4

* 1,250 units streptokinase added to all preparations 1 hour after addition of thrombin. Mixtures then incubated for a further 1-hour period.

† Coagulase positive, strain I.

‡ Defibrinated with glass beads.

during the second hour of the experiment allowed further ingestion to occur as the preparation liquefied. Another possibility is that fibrinogen was removed following the addition of thrombin. If fibrinogen is closely related to phagocytosis, its elimination may have allowed increased phagocytosis. The attempt to measure phagocytosis of (C+) staphylococci in the presence of fibrin only, failed owing to our inability to inactivate neutrophils while fibrinolysis was occurring.

Ingestion of (C+) staphylococci compared with that of (C−) staphylococci. Significantly more (C+) staphylococci were ingested than (C−) staphylococci in all blood preparations. Because fibrin was not a constant factor in determining phagocytosis of staphylococci as measured in the Petri dish technique (owing to fibrinolysis during the second hour of the experiment), the observed differences are most likely the result of chemotaxis.

Ingestion of type II pneumococci. There was a marked increase in the ingestion of encapsulated type II pneumococci in the presence of fibrin strands (blood plus heparin), compared with ingestion of the microorganisms in streptokinase-treated blood or defibrinated blood. The addition of specific antibody to the heparinized blood mixtures resulted in a further increase in encapsulated pneumococcal ingestion. The latter increase was relatively small but under the experimental conditions statistically significant. The addition of specific antibody to the streptokinase-treated or defibrinated blood preparations also resulted in a greatly increased ingestion of encapsulated pneumococci, compared with their ingestion in the absence of antibody. Here too, mean ingestion was somewhat greater than that which occurred in the presence of fibrin only.

Ingestion of rough pneumococci. Statistical analysis of the data on the ingestion of rough pneumococci (variant of the encapsulated type II pneumococcus) in the three blood preparations revealed no clear-cut difference. However, the rough strain was ingested in much greater numbers than the encapsulated strain to which type specific antibody had been added.

Ingestion of (C+) staphylococci compared with that of encapsulated pneumococci. The mean ingestion of (C+) staphylococci was much greater than that of encapsulated pneumococci, especially in the absence of fibrin. These findings show a basic difference in the ability of neutrophils to ingest (C+) staphylococci and encapsulated pneumococci. Whereas, phagocytosis of staphylococcus is relatively easy, the large capsule about the pneumococcus effectively protects against neutrophil ingestion in a fluid medium.

DISCUSSION

The observations reported in the present paper confirm findings of others on the apparent influence of fibrin on the phagocytosis of staphylococci and of pneumococci (1, 2, 5). It appears to play a passive role and influences ingestion differently depending on the microorganism studied. Fibrin probably impedes neutrophil mobility and so limits optimal contact between leucocytes and microorganisms. But actual ingestion of (C+) staphylococci may be hindered by the interposition of a fibrin coat between the staphylococcus and the neutrophil. Encapsulated pneumococci are more readily ingested in a fibrin clot which has been produced by thrombin activity. Here the fibrin strands serve as a barrier against which the microorganisms can be pushed to facilitate ingestion. There is another possible explanation for the differing effect of fibrin on the ingestion of (C+) staphylococci and encapsulated pneumococci. Data presented in this study demonstrated that a significant reduction in phagocytosis of (C+) staphylococci occurred in the presence of fibrin, but failed to show that their ingestion was impossible under these conditions. Neutrophils may be seen to move among fibrin strands in warm stage preparations so it is possible that some ingestion of (C+) staphylococci occurs in fibrin clots. If this is true, the apparent paradox in the role of fibrin on the ingestion of staphylococci and pneumococci could be explained by the fact that both microorganisms are ingested to some extent in the presence of fibrin; but a limiting effect can only be shown with the staphylococcus. Hale and Smith (1) showed that as the exposure time of microorganism to leucocytes in plasma was increased, (C+) staphylococcal ingestion was decreased. They interpret this as evidence that ingestion of (C+) microorganisms occurs before fibrin forms due to coagulase activity, but this experiment is not inconsistent with the above hypothesis.

The formation of fibrin may not be the only factor which influences the ingestion of (C+) staphylococci by neutrophils in plasma. Several investigators (14–16) have reported that (C+) staphylococci clump rapidly when suspended in plasma. This is apparently related to the presence of fibrinogen and usually does not occur in serum; clumping also occurs in fibrinogen solutions (16). If such clumped (C+) staphylococci resisted neutrophil ingestion, consideration of this fact would be required in the interpretation of the results of the present experiments. A suspension of the (C+) strains used in the present study did clump rapidly when tested by the slide-test against two samples of plasma, but they also clumped in sera from the same subjects. The presence of clumping was not looked for in this study so that its effect on the observed phagocytosis cannot be assessed.

The significance of coagulase production in human staphylococcal infections cannot be assessed by the present experiments. The finding of excellent phagocytosis in the presence of fibrin supports the opinion of other investigators (2) that coagulase may not be of prime importance in staphylococcal pathogenicity.

Data presented on the phagocytosis of encapsulated pneumococci in fibrin clots are of great interest in that they permit a quantitative definition of the phenomenon of surface phagocytosis. Exposure of encapsulated type II pneumococci to neutrophils in a fibrin clot resulted in a marked increase in mean ingestion, although antibody was absent. In the presence of type specific antibody mean ingestion was further increased by about 50 per cent. It is clear, however, from these data that "surface phagocytosis" of encapsulated type II pneumococci is nearly as efficient as opsonin-potentiated phagocytosis. This supports the opinion of Wood, Smith, and Watson (4) that "surface phagocytosis" may be an important feature in combatting natural pneumococcal infection prior to the formation of specific antibody.

The influence of streptokinase on the phagocytosis of (C+) staphylococci is not entirely clear. The data show that it does not affect the ingestion of coagulase negative staphylococci, rough pneumococci, or opsonized type II encapsulated pneumococci; but when added to plasma, streptokinase appears to hinder the ingestion of (C+) staphylococci, even though such blood will not clot. The addition of an equal amount of streptokinase to blood defibrinated with glass beads does not influence phagocytosis of the same organisms. All these data suggest that streptokinase does not affect neutrophil activity *per se*. A possible explanation is that the quantity of streptokinase used was insufficient to remove all the fibrinogen from the plasma and so coagulase still promoted the coating of individual staphylococci by fibrin. However, we found no gross or microscopic evidence of fibrin formation following coagulase activity in the preparations.

The observations reported in this paper suggest that the removal of fibrin by streptokinase may be of some value in the local therapy of coagulase positive staphylococcal infection, because mean ingestion was improved over that occurring in heparinized blood. But in pneumococcal infection, it seems likely that streptokinase therapy would impede phagocytosis of encapsulated pneumococci if used to remove fibrin before the appearance of specific antibody.

SUMMARY

1. The production of coagulase by staphylococci diminishes the ingestion of these organisms by neutrophil polymorphonuclear leucocytes.

2. The phenomenon of "surface phagocytosis" of encapsulated pneumococci has been confirmed and defined quantitatively. Mean ingestion by this mechanism was about 65 per cent of that occurring in the presence of specific antibody.

3. Data are presented showing that the removal of fibrin by streptokinase will prevent significant ingestion of encapsulated pneumococci if specific antibody is absent.

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PHAGOCYTOSIS BY HUMAN LEUCOCYTES. II. RELATION OF NUTRITIONAL DEFICIENCY IN MAN TO PHAGOCYTOSIS[1]

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It is generally believed that malnourished patients are more prone to bacterial infection than normal healthy individuals. Increased susceptibility to infection has been attributed to an impaired capacity to produce specific antibodies against the infectious agent (1) or possibly to decreased phagocytic activity resulting from nutritional deficiency (2). Quantitative data have already been presented (3) showing that emaciated patients produce antibody as well as or better than healthy control subjects so that such proneness to develop infection is probably not often related to lack of antibody production. In the present communication studies are reported on the second possibility, namely, that decreased phagocytosis in malnourished patients predisposes them to infection.

The literature relating nutritional states to phagocytosis in animals has already been reviewed by Berry and Spies (4) and need only be considered briefly here. Several groups of workers have suggested, largely on the basis of animal experiments, that nutritional deficiencies, such as specific vitamin deficiencies and protein depletion, may result in decreased phagocytic activity; (5–9) other workers, however, have obtained conflicting results (10–12). It has also been claimed that protein depletion may lead to a diminished output of leucocytes in response to the injection of bacteria or irritants (13, 14).

Few studies on the relationship of nutritional deficiency to the phagocytic activity of human leucocytes are to be found in the literature. Cottingham and Mills (15) reported decreased phagocytosis in 10 malnourished patients, with return to normal as the nutritional states improved; six of these were suffering from acute bacillary dysentery. These investigators suggested that improved phagocytosis might be due to the release of new phagocytes from bone marrow, which had been produced when the nutritional state improved. Berry, Davis, and Spies (16) reported that the phagocytic activity of the neutrophils from patients with mixed dietary deficiency was often 50 per cent or more below control values. On the other hand, Feller, Roberts, Ralli, and Francis (17) noted no significant change in phagocytosis in patients on vitamin A or vitamin C deficient diets. Bieler, Ecker, and Spies (18) noted normal opsonic indices in blood from eight hypoproteinemic, nutritionally deficient patients; and Berry, Leyendecker, and Spies (19) reported increased ingestion in studies on the phagocytic activity of leucocytes in anemic blood. In the latter study many of the subjects were malnourished.

The present paper is a report of *in vitro* studies on the phagocytic activity of neutrophilic polymorphonuclear leucocytes taken from well nourished and severely malnourished patients. This study was carried out in conjunction with the observations reported in the preceding paper. Data with reference to phagocytosis in normal subjects are taken from that paper (20).

METHOD

Phagocytosis was measured in 20 severely malnourished patients and 17 healthy control subjects. The method, utilizing the roller tube technique, has been described in detail in the previous paper. Phagocytosis was measured in both coagulable and non-coagulable blood using a coagulase negative (C−) strain of staphylococcus and four coagulase positive strains (C+I, II, III, IV) used in the previous study (20).

The nutritional state of the patients was determined from the clinical history, by measuring weight loss or gain and by serum protein estimations. Serum albumin and globulin were measured by the Kingsley (21) modification of Howe's method, using 23 per cent sodium sul-

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TABLE I

Ingestion of staphylococci by neutrophils in blood containing heparin or streptokinase or in blood defibrinated with glass beads (Roller tube technique—Malnourished patients)

Blood preparation	Number of subjects	Average neutrophil count per cu. mm.	Number neutrophils counted	Micro-organism*	Mean ingestion per neutrophil	Standard error
Blood plus heparin	20	5,680	4,000	C+	14.6	±1.0
	20		1,000	C−	19.3	±2.0
Blood plus streptokinase	18	6,700	3,600	C+	20.7	±2.2
	17		850	C−	21.7	±2.7
Defibrinated† blood	20	5,800	4,000	C+	30.7	±3.4
	19		950	C−	25.7	±3.5
Defibrinated blood plus streptokinase†	20	5,800	4,000	C+	28.0	±2.7
	20		1,000	C−	18.2	±1.8

* C+ = Coagulase positive strains I, II, III, IV.
C− = Coagulase negative.
† Defibrinated with glass beads.

fate for separation of albumin and globulin. Plasma volume was estimated by the Evans blue dye method, using a Coleman junior spectrophotometer to measure dye concentration. In each case the clinical course was followed carefully and correlated with the above data. The control cases were selected from patients and hospital personnel considered to be well nourished and in good health. Malnourished patients were selected from cases with advanced wasting disease.

No patients received antibacterial drugs while under study.

RESULTS

Ingestion of (C+) and of (C−) staphylococci by neutrophils from malnourished patients

Table I records mean ingestion of (C+) and (C−) staphylococci by neutrophils from malnourished patients. As in the experiments reported in the previous paper, four different blood preparations were used (whole blood plus heparin, whole blood plus streptokinase, blood defibrinated with glass beads, and the latter to which streptokinase had been added). Mean ingestion of all coagulase positive strains (I, II, III, and IV) has been recorded, because phagocytosis was not significantly different with individual strains.

The findings reported in this table are almost identical with those found in the study of normal subjects (20) and may be briefly summarized. There was no significant difference in mean ingestion of (C−) staphylococci in the different blood preparations, so that under the experimental conditions used, heparin, streptokinase, or defibrination of blood with glass beads did not in-

TABLE II

Ingestion of staphylococci by neutrophils in blood from normal and malnourished subjects

Blood preparation	Micro-organism	Normal*		Malnourished*	
		Mean ingestion per neutrophil	Standard error	Mean ingestion per neutrophil	Standard error
Blood plus heparin	C+	15.7	±1.0	14.6	±1.0
	C−	20.4	±1.8	19.3	±2.0
Blood plus streptokinase	C+	23.0	±2.8	20.7	±2.2
	C−	23.7	±2.6	21.7	±2.7
Defibrinated blood	C+	32.7	±1.6	30.7	±3.4
	C−	22.0	±1.7	25.7	±3.5
Defibrinated blood plus streptokinase	C+	33.5	±1.6	28.0	±2.7
	C−	23.9	±1.9	18.2	±1.8

* 17 normal and 20 malnourished subjects.

TABLE III

Phagocytic index related to age and serum proteins—Well nourished subjects

No.	Age	Plasma protein g./100 ml.		Neutrophil count per cu. mm.	Mean ingestion of staphylococci per neutrophil in heparinized blood	
		Total	A/G		Coagulase +	Coagulase −
21	76	6.2	3.4/2.8	2,700	15	25
22	65	7.5	4.1/3.4	4,420	10	19
23	62	7.1	4.2/2.9	2,820	14	14
24	62	5.3	3.4/1.9	1,770	21	32
25	61	7.6	4.3/3.3	4,230	25	31
26	49	—	—	6,640	12	9
27	49	7.3	3.9/3.4	2,380	12	31
28	49	7.2	4.4/2.8	4,680	14	26
29	46	8.0	3.9/4.1	2,900	15	17
30	46	7.4	4.8/2.6	1,660	15	21
31	40	—	—	6,100	8	14
32	37	6.1	4.3/1.8	—	14	16
33	36	6.2	3.7/2.5	2,660	23	24
34	34	6.7	4.7/2.0	—	16	15
35	34	8.1	5.8/2.3	—	14	—
36	29	—	—	6,000	15	11
37	23	6.6	4.4/2.2	—	17	22
Average	47	6.9	4.3/2.7	3,770	15	20

fluence phagocytosis. On the other hand, there was a significant reduction in phagocytosis of (C+) staphylococci associated with the production of fibrin in heparinized blood; mean ingestion of this microorganism was greater in streptokinase-treated blood but still significantly reduced compared with ingestion in blood defibrinated with glass beads.

Comparison of ingestion of (C+) with (C−) staphylococci in heparinized blood and defibrinated blood showed significant differences. Fewer (C+) microorganisms were ingested in heparinized blood while the reverse obtained in defibrinated blood.

Thus, when tested in heparinized blood there was diminished mean ingestion of coagulase-producing staphylococci by neutrophils from malnourished patients. The addition of streptokinase to blood to remove fibrin allowed increased but not maximal ingestion of (C+) staphylococci.

Comparison of phagocytosis in normal and malnourished subjects

Table II lists mean ingestion of staphylococci by neutrophils from normal and malnourished subjects. Comparison of the data showed no significant difference between the groups. The average neutrophil count per cubic millimeter in the malnourished patients ranged from 50 to 90 per cent higher than in the series of normal individuals. Jung and Hanks (*22*, 23) found that as the ratio of leucocytes to microorganisms increased, mean ingestion decreased. This was not found under the experimental conditions used in this study where the ratio of bacteria to neutrophils was approximately 27 : 1. Data relating neutrophil count per cubic millimeter to mean ingestion of the (C+) strain I and the (C−) staphylococcal strain in individual malnourished patients are given in Table V. No consistent relation can be seen, confirming the observations noted in the previous

TABLE IV

Phagocytic index in two well nourished subjects repeated at weekly intervals

	No. 32 Mean ingestion of staphylococci per neutrophil in heparinized blood	
	Coagulase +	Coagulase −
1st week	14	16
2nd week	16	26
3rd week	19	34
4th week	20	18
	No. 34	
	Coagulase +	Coagulase −
1st week	16	15
2nd week	24	39
3rd week	19	27
4th week	32	30

paper (20) and also the findings of another group of investigators (17).

Age and serum protein related to phagocytosis

The age, serum protein, albumin, and globulin levels of well nourished subjects are recorded in Table III. These data are related to mean ingestion of (C+) and (C−) staphylococci. Serum protein levels fell within accepted normal limits, except in patient No. 24; the mean was 6.9 grams per 100 ml. Five of the albumin determinations were below 4.0 grams per 100 ml.; the others were above this arbitrary normal range. Albumin/globulin ratios were normal with one exception, patient No. 29.

Mean ingestion of staphylococci per neutrophil varied considerably. Phagocytosis studied at weekly intervals in two of the well nourished subjects, indicated that the average ingestion per neutrophil was not constant even in a particular individual (Table IV). This is in agreement with the findings of others (17) and points out one of the difficulties in obtaining significant data in a study of this nature.

None of the data recorded in Table III showed any consistent relation to mean ingestion of staphylococci; so, under the conditions studied, age, total serum protein, albumin, and globulin did not seem to be related to phagocytosis in normal subjects.

Age and serum protein determinations of malnourished patients are recorded in Table V. The mean age of the group was 68, considerably higher than that of the normal subjects. Total serum proteins varied widely, but the albumin level was below 4.0 grams per 100 ml. in all but two cases and in 12 of these was below 3.0 grams per 100 ml. Albumin/globulin ratios were reversed in 14 of the patients. As in the well nourished group, there was no consistent relation between the mean ingestion of staphylococci in heparinized blood and the patient's age, the level of total serum protein, albumin, or globulin. Comparison of albumin and globulin means (standard error in each = ± 0.2) in the two groups of subjects revealed a significant difference; but, as previously stated, mean ingestion of staphylococci between the groups was about equal.

The significance of serum protein levels may be difficult to assess from single determinations because of acute or chronic changes in plasma volume; therefore, blood volumes were determined in five unselected patients from each series. Total circulating serum protein, albumin, and globulin

TABLE V

Phagocytic index related to age and serum proteins—Malnourished subjects

No.	Age	Plasma protein g./100 ml.		Neutrophil count per cu. mm.	Mean ingestion of staphylococci per neutrophil in heparinized blood	
		Total	A/G		Coagulase +	Coagulase −
1	86	6.0	2.6/3.4	10,100	10	12
2	83	7.5	4.1/3.4	9,620	15	12
3	78	5.7	2.7/3.0	1,810	19	35
4	77	7.1	3.7/3.4	8,400	10	18
5	76	6.5	3.4/3.1	2,110	16	17
6	74	5.6	2.5/3.1	4,250	18	13
7	74	5.6	2.7/2.9	2,890	20	37
8	72	5.3	2.1/3.2	1,580	7	6
9	71	7.5	2.9/4.6	8,130	14	23
10	71	6.4	1.7/4.7	6,320	15	20
11	70	7.3	3.1/4.2	2,160	13	28
12	67	6.2	2.7/3.5	—	9	10
13	65	4.4	2.0/2.4	6,340	10	17
14	64	7.5	2.5/5.0	4,070	12	11
15	63	8.3	3.7/4.6	5,900	16	20
16	63	5.5	3.3/2.2	3,460	21	27
17	63	6.1	2.0/4.1	2,280	19	29
18	56	—	—	21,000	6	11
19	54	5.6	2.2/3.4	5,100	16	33
20	32	6.7	4.0/2.7	2,680	21	27
Average	68	6.4	2.8/3.5	5,680	14	20

TABLE VI

Phagocytic index related to total circulating plasma protein, plasma albumin, plasma globulin and total red cell mass

Patient number	Age	Plasma protein g./100 ml.	Total circulating			Total red cell mass ml.	Approximate number circulating neutrophils $\times 10^9$	Mean ingestion of staphylococci per neutrophil in heparinized blood	
			Plasma protein g.	Plasma albumin g.	Plasma globulin g.			C+	C−
Well nourished group									
23	62	7.1	242	143	99	2,750	21	14	14
24	62	5.3	201	129	72	4,090	15	21	32
27	49	7.4	204	109	95	2,895	18	12	31
28	48	7.2	268	163	105	2,660	38	14	26
29	46	8.0	395	195	200	3,620	29	15	17
Mean	53	7.0	262	147	114	3,200	24	15	24
Malnourished group									
3	78	5.7	180	87	93	1,150	9	19	35
6	74	5.6	163	73	90	2,480	28	18	13
9	71	7.5	190	75	115	1,800	40	14	23
13	65	4.4	113	50	63	1,330	27	10	17
16	63	5.5	145	88	57	1,730	19	21	27
Mean	70	5.7	158	75	84	1,700	25	16	23

were calculated and are recorded in Table VI. The mean value for total protein and albumin was 60 and 51 per cent lower in the malnourished patients; but mean ingestion of staphylococci in the two groups was not significantly different.

Anemia and phagocytosis

Berry and his associates (19, 24–26) have reported that leucocytes from anemic blood show more active phagocytosis than those obtained from patients with normal red cell counts. It is believed by these authors that the neutrophils from anemic blood have undergone some fundamental change which did not appear related to increased motility but which endowed them with greater phagocytic capacity than normal cells. Table VI records the total circulating red cell mass of five patients from each of the nutritional groups. The difference between the two groups is marked, but this is not reflected in any significant difference in phagocytosis. Therefore, under the conditions of the present experiments, there was no significant difference in the phagocytic activity of leucocytes taken from anemic and normal patients.

Total circulating neutrophils in normal and malnourished subjects

Table VI also records a rough approximation of the number of circulating neutrophils present in the five normal and five malnourished subjects. These were calculated by determining neutrophil count per cubic millimeter and multiplying this figure by the known blood volume. The error inherent in these estimations was probably of the same order in the two groups of patients. There was no significant difference in numbers of neutrophils between the groups.

Clinical state of subjects studied

A brief description of the clinical picture presented by the malnourished patients is recorded in Table VII, which indicates the severe degree of disease present. Findings commonly associated with far advanced wasting disease, such as extensive weight loss, emaciation, decubitus ulcers, asthenia, and anemia, were present in many of the patients. Blood was taken for study from several patients within three days of death.

DISCUSSION

The data presented in this paper show that there was no significant difference in the mean phagocytosis of staphylococci by neutrophils from the normal and malnourished patients studied. The mean age of the two groups was different, but there was no evidence that patient age is a factor in phagocytosis. The microorganisms used in the study were selected for two reasons: 1) Staphylococci may be associated with infections in mal-

TABLE VII

*Diagnosis and clinical course in malnourished patients**

Patient No.	
1.	Carcinoma of head of pancreas with obstructive jaundice; 2-year history; patient emaciated and terminal in appearance; died. (10)
2.	General and cerebral arteriosclerosis; prostatic hypertrophy; patient confined to room for 3 years with inadequate care; emaciated; discharged to State Mental Institution.
3.	Femoral neck fracture; chronic alcoholism with portal cirrhosis; transferred unimproved.
4.	Emaciated patient admitted to hospital in terminal state; unable to eat; undiagnosed; died. (1)
5.	Carcinoma of sigmoid colon; admitted with intestinal obstruction requiring cecostomy followed by transverse colostomy. Progressive weight loss; transferred to State Mental Institution, unimproved.
6.	Metastatic carcinoma of liver with 80-pound weight loss during preceding 12 months; repeated massive rectal hemorrhages; died. (3)
7.	Adenocarcinoma of rectum with metastases. Progressive weight loss and emaciated in appearance. Apparently terminal; died. (2)
8.	Chronic alcoholism with malnutrition and 20-pound weight loss; leg ulcers; improved with adequate diet.
9.	Carcinoma of esophagus; history of progressive dysphagia for 1 year; 50-pound weight loss; decubitus ulcers and peripheral edema; died. (8)
10.	Laennec's cirrhosis with ascites and peripheral edema; weight loss; discharged unimproved.
11.	Emaciated patient with large decubitus ulcers admitted in uremia; died without a diagnosis. (2)
12.	Adenocarcinoma of rectum with metastases to bladder, peritoneum and liver; ascites; discharged unimproved.
13.	Probable carcinoma of stomach with pyloric obstruction. Pulmonary tuberculosis; died. (3)
14.	Amyotrophic lateral sclerosis with 30-pound weight loss and large decubitus ulcers. Unimproved. (42)
15.	Unknown.
16.	Colonic diverticulosis. History of chronic diarrhea of 3–4 years' duration; 30- to 40-pound weight loss. Discharged improved.
17.	Laennec's cirrhosis with ascites and peripheral edema. Transferred unimproved.
18.	Carcinoma of lung; extremely wasted with extensive decubitus ulcer; course rapidly deteriorating. Transferred unimproved.
19.	Carcinoma of lung with metastases; 40- to 45-pound weight loss; progressive deterioration. Discharged unimproved.
20.	Benign stricture of esophagus requiring gastrostomy. Best weight 105 pounds, present weight 71 pounds. Discharged improved.

* Figures in parentheses indicate interval (in days) between determination of phagocytic index and death.

nourished patients; 2) It seemed possible that a defect in phagocytosis might appear only if a microorganism was used which was somewhat resistant to ingestion. This was investigated by comparing the ingestion of both (C+) and (C−) staphylococci in heparinized blood; diminished ingestion might then have become apparent only with the (C+) strain.

The data show that under the experimental conditions neutrophil count did not significantly influence mean ingestion; therefore, the fact that the mean neutrophil count was higher in the malnourished group does not alter the significance of the comparison of phagocytic indices found.

There can be little doubt that the patients included in the malnourished group were severely wasted. Hypoproteinemia was a prominent feature in many of the cases although in some of these starvation was not the only factor. Carcinoma with metastases was the primary cause of death in nine of the patients. Cachexia, associated with carcinoma, may be due to several causes, so it is possible that the high phagocytic indices found in such cases may not truly represent the state of neutrophil activity likely to be present in uncomplicated malnutrition. Nevertheless, phagocytic indices were normal in two of the three patients studied (numbers 2 and 20) in whom malnutrition was due to simple starvation; and they were also normal in the three patients with portal cirrhosis of the liver, a lesion thought to be associated with inadequate diet. Blood for study was drawn within three days of death from wasting disease in five of the patients; yet mean ingestion by the neutrophils was normal. It seems probable, therefore, that phagocytosis of staphylococci by neutrophils may be normal regardless of the nutritional state of a patient, provided maturation has occurred and a suitable environment is present.

The possibility remains that malnourished patients may not have the capacity to produce sufficient numbers of normal neutrophils in response to a prolonged stimulus. The data presented in Table VI showing the approximate number of circulating neutrophils in normal and malnourished patients are of interest, although probably insufficient from which to draw general conclusions. In four of the malnourished patients (numbers 6, 9, 13, and 16), the total circulating neutrophil count was comparable to control values; moreover, the mean ingestion by these neutrophils was also comparable. The first three of these patients were among the most severely wasted of the entire group, disease having been present for several months. In two of the patients, blood was drawn for study three days before death and in the third, eight days before death. These cases show quite

definitely that a patient may possess normal numbers of viable neutrophils in the circulation following prolonged starvation.

The findings of this study are not comparable with some of the other reported studies on malnutrition and phagocytosis (5–9). Much of the previous work has been done with leucocytes from animals which have been on protein or specific vitamin deficient diets; furthermore, staphylococcus was not always used to test ingestion. The results of the present study cannot be compared with those reported in a series of patients with acute dysentery (15), because no patient with that diagnosis was included. Furthermore, the techniques used were not identical, and phagocytic activity in the dysentery patients was reported only as the per cent of that of two normal controls. It is possible that the severe changes in intravascular water and electrolyte content which are frequently present in acute dystentery may have altered the environment necessary for optimal phagocytosis. The improvement in phagocytosis recorded as the symptoms of dysentery abated may have been a reflection of restoration of the normal plasma volume. The findings of this paper agree with one study (19) and disagree with another (16) found in the literature on phagocytosis in malnourished patients.

The present studies failed to show increased phagocytosis in relation to anemia. Berry and Spies (4) were only able to show such a relationship by adjusting the *in vitro* phagocytic system so that the number of leucocytes in anemic and normal blood was comparable. Our findings may be different, therefore, because no attempt was made to control this variable, although the experiments did not show leucocyte count to be an important factor. Another possible explanation for the failure to confirm the findings of the above investigators is that a larger ratio of microorganisms to leucocytes was used in the present study. Because mean ingestion in our controls was relatively large, the technique may not have permitted the demonstration of any further increase.

In a previous paper quantitative evidence was presented to show that patients may still produce large amounts of antibody within a few days of terminal wasting disease (3). The present report shows that similar patients may also produce normal quantities of neutrophils shortly before death which can ingest staphylococci with ease. Therefore, the increased incidence of infection found in malnourished patients is probably not often related to a defect in the above defense mechanisms.

SUMMARY

1. Phagocytosis of four coagulase positive and one coagulase negative staphylococcal strains has been studied in 17 well nourished subjects and in 20 patients with advanced wasting disease.

2. There was no significant difference in the ingestion of staphylococci by the neutrophil polymorphonuclear leucocytes obtained from these two groups. Phagocytosis could not be related to serum protein levels, total red cell mass, or age of the patient.

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THE PHYSIOLOGIC EXPLANATION OF THE CHANGES IN THE CORONARY CIRCULATION PRECIPITATED BY AORTIC-CORONARY SINUS ANASTOMOSIS [1, 2]

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In recent years we have been able to confirm the observations made by Hahn and Beck (1) in demonstrating a significant protection against ventricular fibrillation following coronary artery ligation in dogs in which a chronic aortico-coronary sinus anastomosis has been produced (2). Our recent interest lies in the physiologic explanation of this protection. Accordingly, we have outlined a series of experiments to elucidate two major points with respect to chronic arterialization of the coronary sinus in dogs: 1) whether retrograde blood flow occurs; and 2) if retrograde blood flow does occur, whether or not this blood first traverses a capillary bed. The following information was therefore first secured in normal control dogs: 1) coronary sinus pressure and oxygen content of coronary sinus blood; 2) quantitative determination of retrograde blood flow following circumflex coronary artery ligation from a point distal to the ligature; and 3) determination of oxygen content and saturation of blood samples taken simultaneously from the pulmonary artery, aorta, and the distal end of the cut circumflex coronary artery. Inasmuch as any operation on the heart, by producing pericardial adhesions, might be presumed to alter these findings, we felt it essential to repeat these experiments in dogs having previously had nondescript cardiac procedures. When possible, dogs were used in which unsuccessful arterialization of the sinus had been previously performed, with subsequent thrombosis of the graft. These experiments were repeated in dogs in which successful arterialization of the sinus had been performed. Moreover, in these dogs coronary sinus pressure, retrograde flow from the distal cut end of the ligated coronary artery, and oxygen content data were obtained with the graft open and closed.

METHODS

Group A constituted the normal control, and consisted of ten mongrel dogs whose weights varied between 11 and 17 kilograms. They were anesthetized with 0.5 cc. of halatal per kg., after which artificial respiration was maintained using intermittent positive pressure. The left chest was opened between the fourth and fifth ribs, the pericardium was opened, and the coronary sinus was cannulated. The cannula was connected to a water manometer, and the sinus pressure and the specimen of coronary sinus blood were obtained. The circumflex branch of the left coronary artery was isolated at its origin and ligated. An incision was made in the artery just distal to the ligature, through which the distal artery was cannulated and specimens of retrograde coronary artery blood were obtained. Simultaneously, aortic and pulmonary arterial blood were also obtained. The minute volume of retrograde bleeding from the distal cut end of the circumflex artery was recorded. The oxygen content of all blood samples was determined according to the method of Van Slyke and Neill (3). Oxygen capacity and saturation were determined in the sample of aortic blood.

Group B consisted of ten mongrel dogs of similar weight on which other forms of cardiac surgery had been previously performed. In four of these dogs a previous attempt at arterialization of the coronary sinus had been made, but the grafts had been demonstrated to be thrombosed and non-functioning. In the remaining dogs the right atrium had previously been opened for a period of six to twelve minutes during hypothermia experiments conducted one to three months previously. In some of the dogs in this group, interatrial septal defects had been created and closed. In five of the dogs, the anterior descending branch of the left coronary artery was ligated at its origin, and in the remaining five, the circumflex

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branch of the left coronary artery was ligated. The distal segment of the ligated vessel was then cannulated, and samples were obtained similar to those in Group A.

Group C consisted of ten mongrel dogs which were first prepared with arterialization of the coronary sinus in a two stage operation. At a first stage, through a left-sided approach, a segment of an external jugular vein was used to anastomose the aorta to the coronary sinus. Three to four weeks later, through a right-sided approach, the coronary sinus was partially ligated, over a probe, to a residual diameter of two or three millimeters. Four to eight weeks following the second stage of the procedure, the animals were anesthetized with halatal, after which artificial respiration was maintained by using intermittent positive pressure through an endotracheal tube. The left chest was entered through the fourth intercostal space and all pleural and pericardial adhesions were divided. The vein graft was dissected and examined for patency. Only dogs with functioning grafts were used. An umbilical tape was passed around the graft to permit easy occlusion at the appropriate time during the procedure. A needle-tipped polythene catheter connected to a water manometer was inserted into the coronary sinus, after which sinus pressure and blood samples were obtained. The circumflex branch of the left coronary artery was carefully dissected to its origin and ligated. The artery was cannulated distal to the ligature and a sample of the retrograde blood was obtained, simultaneous samples being obtained from the aorta and the pulmonary artery. The timed minute volume of retrograde bleeding was then immediately recorded (Figure 1). Then the graft was clamped, and the experiment was repeated (Figure 2). This was the method used for the first five dogs studied.

During the study of the sixth dog, following the clamping of the vein graft, the cannula inadvertently slipped out of the circumflex artery distal to the ligature. It was noted that the retrograde bleeding was dark prior to the clamping of the graft, and that it remained so immediately after clamping. Several minutes elapsed before the cannula was reinserted into the artery during all of which time the graft was clamped. It was noted that during the period of time necessary for the reinsertion of the cannula, the retrograde blood changed from blue to red. Consequently, in dogs 6 to 10, after the graft had been clamped, the circumflex artery was permitted to bleed freely for a period of two minutes prior to obtaining blood samples for oxygen content.

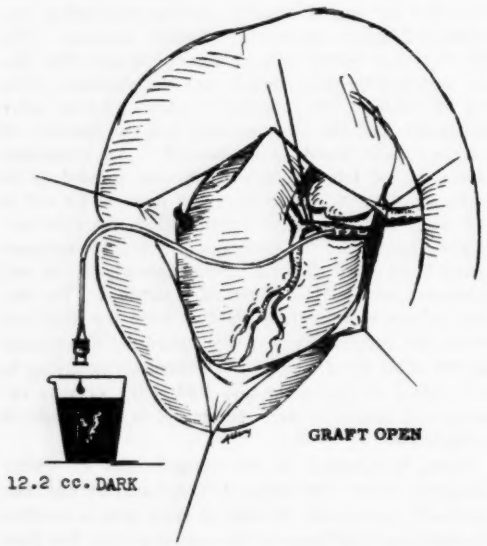

FIG. 1. CANNULATING THE DISTAL SEGMENT OF THE LIGATED LEFT CIRCUMFLEX CORONARY ARTERY

The circumflex branch of the left coronary artery is isolated at its origin, and ligated. An incision is made in the artery just distal to the ligature, through which the distal artery is cannulated. Retrograde minute blood flow, and blood samples for determination of oxygen content are obtained from the distal cut end of the circumflex branch of the left coronary artery in this fashion.

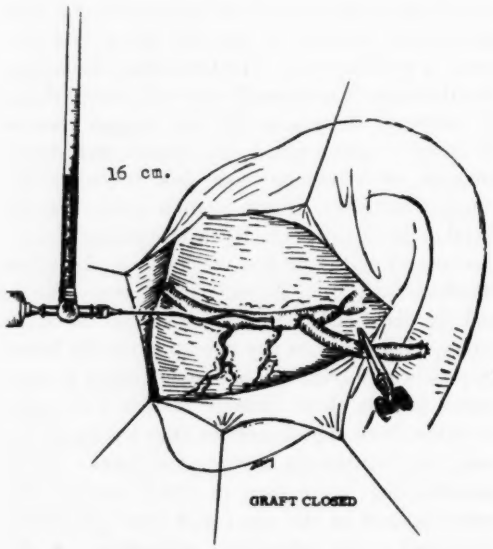

FIG. 2. MEASUREMENT OF CORONARY SINUS PRESSURES

A needle tipped polythene catheter is inserted into the coronary sinus proximal to its point of partial ligation, and is attached to a water manometer. The graft is clamped, and the fall in coronary sinus pressure is recorded. Following a two-minute interval, after clamping of the graft, a blood specimen is obtained for determination of oxygen content.

TABLE I

Retrograde blood flow and oxygen content of blood from the distal cut end of the ligated left circumflex coronary artery in normal dogs *

Dog No.	Retrograde cor. art. Flow cc./min.	Retrograde cor. art. O_2 content vol. %	Aortic blood Content vol. %	Aortic blood Cap. vol. %	Pul. art. O_2 content vol. %	Coronary sinus Press. cm. water	Coronary sinus O_2 content vol. %	Survival time
1	2.2	17.3	18.8		10.2	12	9.2	30 min.
2	1.4	15.1	15.8	16.8	12.6	4	6.3	36 hours
3	4.4	17.5	18.2	18.2	12.2	2	6.7	18 min.
4	1.0	14.7	16.2	16.7	10.9	5	4.4	20 min.
5	0.4	15.8	16.9	17.8	14.5	8.6	2.9	3 min.
6	0.5	18.0	17.5	17.8	11.5	8.4	7.4	4 min.
7	0.6	15.4	16.3	17.1	11.6	4.6	3.6	15 min.
8	1.1	8.1	7.2	12.2	4.8	4.0	1.0	16 min.
9	1.0	11.6	11.5	17.4	8.6	7	2.4	5 min.
10	5.0	11.9	14.7	19.5	9.5	5	1.3	12 min.
Aver.	1.7	14.5	15.3		10.6	6.1	4.5	

* Death was by ventricular fibrillation in all except Dog 2.

RESULTS

A. *Retrograde bleeding from the distal segment of the divided circumflex branch of the left coronary artery*

1) *Group A:* The retrograde bleeding ranged from 0.4 to 5.0 cc. with an average of 1.7 cc. per minute (Table I).

2) *Group B:* Retrograde bleeding from the distal segment of the divided circumflex branch of the left coronary artery ranged from 0.5 to 6.0 cc., with an average of 1.9 cc. per minute (Table II). Retrograde bleeding from the left anterior descending artery ranged from 1.8 to 11.0 cc. with an average of 5.1 cc. per minute (Table II). Two of the dogs in which the left anterior descending branch was ligated presented a large accessory anterior descending branch arising from the circumflex artery. This accessory branch may have accounted for the excessive retrograde bleeding by providing additional intercoronary communications.

3) *Group C:* With the graft open, retrograde bleeding from the left circumflex artery ranged from 9.0 to 18.0 cc. per minute with an average of 12.2 cc. per minute. After the vein graft was clamped, the sinus was to all intents and purposes no longer arterialized. At this point, the retrograde bleeding from the circumflex artery fell significantly in each dog. The retrograde bleeding with the graft closed ranged from 2.0 to 10.0 cc. with an average of 7.1 cc. per minute (Table III).

B. *Oxygen content of the retrograde blood from the circumflex artery, obtained from the distal cut end of the vessel*

1) *Group A:* Although blood from the aorta, pulmonary artery and distal segment of the coronary artery varied from dog to dog, the retrograde coronary arterial blood was always highly oxygenated, and its oxygen content usually approximated that of aortic blood, ranging from 8.1 to 18.0 volumes per cent, with an average of 14.5 volumes per cent. Concomitant samples of aortic blood ranged from 7.2 to 18.8 volumes per cent, with an average of 15.3 volumes per cent (Table I).

2) *Group B:* In this group, the findings were similar to those in Group A. Oxygen content varied from dog to dog, but the blood was always highly oxygenated, and except for animal No. 5, approximated that of aortic blood, ranging from 11.4 to 24.0 volumes per cent with an average of 16.6 volumes per cent. Concomitant samples of aortic blood ranged from 10.7 to 23.4 volumes per cent with an average of 16.5 volumes per cent (Table II).

3) *Group C:* The oxygen content of blood obtained from the distal cut end of the circumflex artery in this group again varied widely from dog to dog. The oxygen content of the retrograde blood ranged from 1.5 to 15.1 volumes per cent, with an average of 6.3 volumes per cent. Concomitant samples obtained from the aorta ranged from 12.1 to 22.5 volumes per cent, with an average

TABLE II

Retrograde blood flow and oxygen content of blood from the distal cut end of the ligated left coronary artery in dogs having had nondescript cardiac surgery

Dog No.	Retrograde cor. art.		Aortic blood	Pul. art.	Coronary sinus		Remarks
	Flow *cc./min.*	O_2 content *vol. %*	O_2 content *vol. %*	O_2 content *vol. %*	Press. *cm. water*	O_2 content *vol. %*	
Left circumflex coronary artery							
1	1.1	17.3	16.4	12.2	5.4		Thromb. Beck D. 6 wks.
2	1.2	24.0	23.4	20.6	6.0		Thromb. Beck D. Vent. Fib. 20 min.
3	1.2	11.4	10.7	4.6	23		I.A. septal defect under hypoth. cong. fail. D. card. arrest 17 min.
4	6.0	17.8	18.0	11.9	12		Thromb. Beck D. Vent. Fib. 20 min.
5	0.5	14.0	17.5	10.6	6.4	4.8	Hypoth. D. 14 min. Vent. Fib.
6	1.4	18.3	17.2	10.1	4.0	4.3	Thromb. Beck D. 48 hours
Aver.	1.9	17.1	17.2	11.6	6.7	4.5	
Left anterior descending coronary artery							
7	1.8	13.6	15.0	10.0			Rt. aur. open. under hypoth. D. 10 min. Vent. Fib.
8	1.5	14.9	14.9	10.1			I.A. septal defect under hypoth. D. 23 min. Vent. Fib.
9	11.0	18.0	17.1	13.0			I.A. septal defect under hypoth. large access. L.A.D. from circum. Lived 30 days till sacrif.
10	6.2	17.2	14.5	10.5			I.A. septal defect under hypoth. access. L.A.D. Lived 30 days till sacrif.
Aver.	5.1	15.9	15.1	10.9			

of 17.1 volumes per cent. Despite the wide range of values in the oxygen content of the retrograde circumflex blood, the latter was constantly venous in nature, and its content was always lower than that of pulmonary arterial blood (Table III). Moreover, the oxygen content of the retrograde blood was within the limit of that normally expected for coronary sinus blood (Table I). In fact, the average oxygen content of the retrograde coronary arterial blood was 6.3 volumes per cent, which compared favorably with the average oxygen content of normal coronary sinus blood, which was 4.5 volumes per cent.

Following clamping of the graft, the values ob-

TABLE III

Retrograde blood flow and oxygen content of blood from the distal cut end of the ligated left circumflex coronary artery in sinus arterialized dogs with the graft open and closed

Dog No.	Graft	Retrograde cor. art. Flow cc./min.	Retrograde cor. art. O_2 content vol. %	Coronary sinus Press. cm. H_2O	Coronary sinus O_2 content vol. %	Aortic blood Cont. vol. %	Aortic blood Cap. vol. %	Aortic blood Sat. %	Pul. art. O_2 content vol. %	Remarks
1	Open	9.6	9.0	45		15.2			10.3	8 wks. P. 2nd st. D. Tens. Pneumo. 24 hrs.
2	Open	9.0	1.9	80	17.2	18.8	20.6	91.3	11.4	12 wks. P. 2nd st. D. Tens. Pneumo. 10 hrs.
	Clos.	7.0	5.3							
3	Open	11.0	11.8	60	12.5	19.2	20.7	92.8	15.9	4 wks. P. 2nd st. D. Hemor. 24 hr. PO.
4	Open	12.0	6.1	36	12.4	14.0	15.8	99.6	11.1	5 wks. P. 2nd st. D. 3 min. P. Graft Closed. Card. arrest
	Clos.	10.0	7.7							
5	Open	12.0	1.5	60	9.7	12.1	13.0	93.1	10.2	5 wks. P. 2nd st. D. 5 min. P. Graft Closed. Vent. Fib.
	Clos.	7.0	4.6	20						
6	Open	10.0	4.8	71	15.5	17.7	18.4	96.2	14.3	4 wks. P. 2nd st. D. 96 hrs. Pneumo. and Emp.
	Clos.	2.0	12.5	9						
7	Open	18.0	4.0	64	11.9	14.3	14.0	100	9.2	4 wks. P. 2nd st. Lig. L.A.D. Vent. Fib.
	Clos.	8.0	13.2	19	7.1					
8	Open	10.0	15.1	59	17.9	22.5	22.5	100	19.1	4 wks. P. 2nd st. No Ad'hsn's. Surv. Lig.
	Clos.	8.0	18.5	18						
9	Open	14.0	4.8	68	13.2	17.2	20.1	85.6	7.6	8 wks. P. 2nd st. D. 6 hrs. Tens. Pneumo.
	Clos.	7.0	15.3	16	1.7					
10	Open	16.0	3.9	70	15.6	17.8	19.8	89.9	11.6	6 wks. P. 2nd st. D. 12 hrs. Tens. Pneumo.
	Clos.	8.0	17.9	14	1.9					
Average:	Open	12.2	6.3	61.3	14.0	17.1			12.1	
	Clos.	7.1	15.5	16.0	3.6					

tained for the oxygen content of retrograde circumflex arterial blood divide themselves into two groups. The first group consists of those animals in which the blood was collected immediately after the clamping of the graft (dogs 1–5, Table III). The second group consists of those animals in which the ligated circumflex artery was permitted to bleed freely in retrograde fashion for a period of two minutes prior to the collection of the specimens (dogs 6–10, Table III). Inasmuch as these values are markedly different, and we believe this difference to be significant, we are reporting them separately. In the first group the oxygen content of the retrograde blood ranged from 4.6 to 7.7 volumes per cent with an average of 5.8 volumes per cent. This, it may be noted, is almost identical to the average oxygen content of retrograde blood with the graft open. However, the data from individual dogs indicate that in each case there was significant rise in the oxygen content of the circumflex retrograde arterial blood immediately after clamping of the graft. In dog 2, oxygen content of the retrograde blood with the graft open was 1.9 volumes per cent, and rose promptly to 5.3 volumes per cent. In dog 4, the value rose from 6.1 to 7.7 volumes per cent; in dog 5, from 1.5 to 4.6 volumes per cent. It is possible that had the artery been permitted to bleed

freely in retrograde fashion for a period of two minutes, the values obtained in dogs 1–5 would have risen further towards that of the content of systemic arterial blood, as occurred in animals 6–10.

In the second group of animals (6–10) the oxygen content of the blood ranged from 12.5 to 18.5 volumes per cent, with an average of 15.5 volumes per cent. Despite the wide range of values, the blood was compatible in oxygen content to the aortic blood. Concomitant samples of aortic blood in dogs 6–10 ranged from 14.3 to 22.5 volumes per cent, with an average of 17.9 volumes per cent. The blood oxygen content obtained from the retrograde flow of the left circumflex artery increased significantly from an average of 6.3 volumes per cent with the graft open, to an average of 15.5 volumes per cent with the graft closed, and changed in appearance from dark venous to bright arterial blood.

C. *Mean coronary sinus pressure and oxygen content of coronary sinus blood*

1) *Group A:* The mean coronary sinus pressure ranged from 2 to 12 cm. of water with an average of 6.1 cm. of water. The coronary sinus blood oxygen content ranged from 1.0 to 9.2 volumes per cent, with an average of 4.5 volumes per cent (Table I).

2) *Group B:* The mean coronary sinus pressure ranged from 4.0 to 12 cm. of water with an average of 6.7 cm. of water. The one dog with a mean coronary sinus pressure of 23 cm. of water is not included in this average since it was in severe right heart failure, and was included to determine the effect of elevated coronary sinus pressure without sinus occlusion upon retrograde coronary artery bleeding. Coronary sinus blood oxygen content determined in two dogs, was 4.8 and 4.3 volumes per cent, respectively (Table II).

3) *Group C:* With the graft open, coronary sinus pressure ranged from 36 to 80 cm. of water with an average of 61 cm. of water. The oxygen content of the blood obtained from the arterialized coronary sinus ranged from 9.7 to 17.9 volumes per cent with an average of 14.0 volumes per cent. There was no direct relationship between the quantity of retrograde bleeding and mean coronary sinus pressure (Figure 3).

Following the clamping of the graft, the coronary sinus pressure fell significantly towards, but not quite to normal sinus pressure, as established in the normal dogs. These pressures ranged from 9 to 20 cm. of water, with an average of 16 cm. of water. In a few dogs, specimens of coronary sinus blood were obtained two minutes after clamping of the graft. The oxygen content of these samples ranged from 1.7 to 7.1 volumes per cent, with an average of 3.6 volumes per cent. Thus, in these animals, the oxygen content of the coronary sinus blood fell from within arterial range with the graft open, to oxygen contents within normal sinus range following clamping of the graft.

DISCUSSION

The most striking result was the marked difference in the amount and nature of the retrograde bleeding from the distal cut end of the circumflex artery in normal and in sinus arterialized dogs. In all instances the retrograde blood approximated the oxygen content of blood simultaneously obtained from the aorta, and probably originates in the non-occluded coronary arteries by intercoronary arterial collateral flow.

We have demonstrated that nonspecific cardiac procedures *per se* alter neither the quantity nor the character of the blood flowing in retrograde fashion from the coronary artery of the normal animal. This change in the character and the quantity of the retrograde blood apparently only follows arterialization of the coronary sinus. These findings attest to the increase in the retrograde flow through the circumflex artery following aortico-coronary sinus anastomosis, and the venous nature of this blood is evidence that it has traversed the myocardial capillary bed, with the extraction of oxygen by the myocardium.

This increase in retrograde flow has been similarly demonstrated by Hahn, Kim, and Beck (4), and Eckstein and his associates (5–8). The latter have similarly demonstrated a retrograde perfusion of the capillary bed of the occluded circumflex artery, acutely, and for about one month after coronary sinus arterialization.

An almost control situation has been obtained shortly after the graft has been occluded. The coronary sinus pressure falls significantly towards, but not quite to normal levels, whereas the retro-

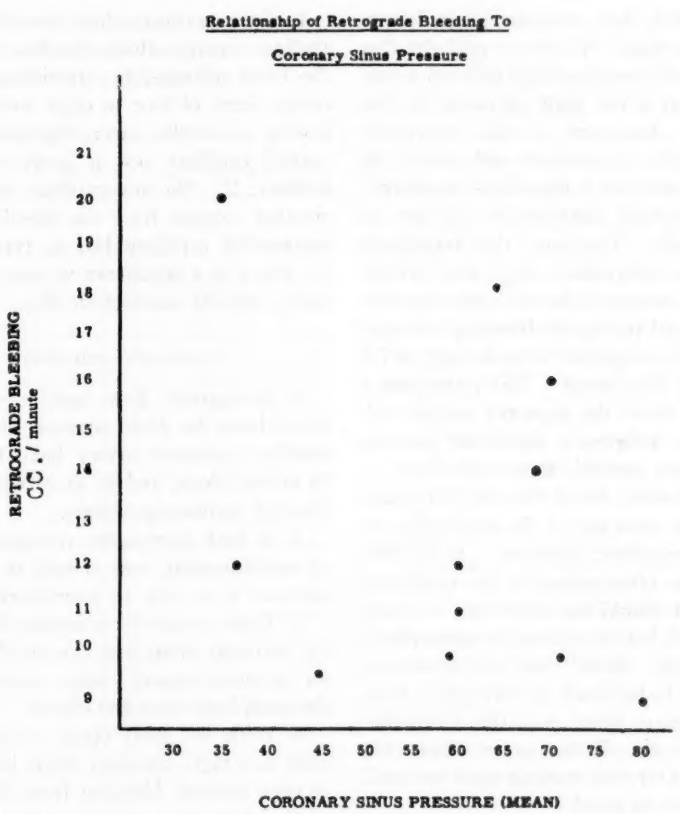

FIGURE 3

The graft shows no noticeable relationship between retrograde bleeding and mean coronary sinus pressure in dogs in which the sinus was chronically arterialized for four to eight weeks.

grade blood becomes arterial in nature, both simulating those values found in the normal dog. It may be of significance that the pressure in the coronary sinus does not fall to normal.

Gregg, Dewald, Thornton, and Mautz (9–12) have demonstrated that, following ligation of the coronary sinus, without preceding arterialization, retrograde bleeding from the distal cut end of the descending ramus of the left coronary artery increased in quantity up to 39 cc. per minute, and became venous in nature. It may be inferred that the increased residual coronary sinus pressure may be a factor in the increased retrograde bleeding from the circumflex artery following occlusion of the graft. Eckstein, Hornberger, and Sano (6) have shown that the retrograde bleeding in dogs in which the coronary sinus was arterialized acutely varied proportionately with the pressure in the coronary sinus. This relationship was not noted in dogs in which the sinus was chronically arterialized for four to eight weeks (Figure 3). Moreover, in several animals in which the coronary sinus pressure was elevated to 48 to 58 cm. of water by complete occlusion of the coronary sinus proximal to its entrance into the right atrium, retrograde flow averaged approximately 2.5 cc. Though coronary sinus pressure was elevated 8 to 10 times above normal, the flow from the distal segment of the divided circumflex coronary artery was within normal range (13).

Following occlusion of the graft, the volume of retrograde bleeding from the distal cut end of

the circumflex fell, but remained significantly elevated above normal. It seems unlikely that the residual elevated coronary sinus pressure alone, following occlusion of the graft, accounts for this retrograde flow. Inasmuch as this retrograde bleeding was highly oxygenated and arterial in nature, it is probable that it represents blood arising from intercoronary anastomoses, as seen in the normal animals. However, this retrograde bleeding in sinus arterialized dogs was greater than the normal amount of intercoronary anastomotic flow. Normal retrograde bleeding averaged 1.7 cc. per minute, as opposed to an average of 7.1 cc. per minute in this group. This represents a fourfold increase above the expected normal values, and probably indicates a significant increase in the intercoronary arterial anastomotic flow.

In these experiments, blood flows in retrograde direction from the open end of the circumflex artery, against atmospheric pressure. It is difficult to relate these observations to the conditions which would exist should the circumflex coronary artery be occluded, but not opened to atmospheric pressure. Probably, under these circumstances, the capillary bed is perfused in retrograde fashion from the coronary sinus, with this blood then being drained *via* the Thebesian or other deep venous systems. Our observations must be qualified with this point in mind.

In the present experiments on dogs with arterialized coronary sinus, the heart continued to beat in vigorous, uninterrupted fashion, following coronary artery ligation. Whereas it was necessary to proceed rapidly to obtain data in the normal animals prior to the onset of ventricular fibrillation, we soon learned that we could proceed slowly in the sinus arterialized animals, provided the graft was left open. However, when the graft was occluded, it was necessary to obtain the data with dispatch lest the ventricles fibrillate with abrupt cessation of the retrograde arterial blood flow. In several sinus arterialized dogs in which the heart beat well for considerable periods of time following circumflex artery ligation, ventricular fibrillation ensued within 5 minutes of clamping of the graft. This finding further emphasized the value of the protection afforded by the aortico-coronary sinus anastomosis. These observations are in accord with those found by others (1, 4, 5, 14).

In the experiments here described, three salient findings emerge which elucidate the protection to the heart afforded by arterialization of the coronary sinus, of four to eight weeks duration, following circumflex artery ligation: 1) The myocardial capillary bed is perfused in retrograde fashion; 2) The myocardium is capable of extracting oxygen from the blood supplied to the myocardial capillary bed in retrograde fashion; 3) There is a significant increase in the intercoronary arterial anastomotic flow.

SUMMARY AND CONCLUSIONS

1. Retrograde flow and oxygen content of blood from the distal segment of the divided circumflex coronary artery have been determined in normal dogs, and in those with previous nondescript cardiac operations.

2. In both groups, the retrograde bleeding was of small quantity, was arterial in nature, and was believed to be due to interarterial collaterals.

3. These studies were repeated in dogs in which the coronary sinus was chronically arterialized by an aortico-coronary sinus communication, with the graft both open and closed.

4. With the graft open, coronary sinus pressure was high, coronary sinus blood was of high oxygen content, bleeding from the distal cut end of the circumflex coronary artery was greater in quantity than normal, and of low oxygen content. This demonstrated a reversal of blood flow through the capillary bed, with extraction of oxygen by the myocardium.

5. With the graft occluded, coronary sinus pressure fell significantly towards normal, and the blood oxygen content fell to control levels. Concomitantly, retrograde circumflex arterial blood oxygen content rose to arterial levels, and its flow fell significantly. This bleeding was still four times greater than the expected normal, and was believed to represent a significant increase in the interarterial coronary collateral anastomoses.

6. The physiologic explanation of the protection to the heart afforded by anastomosis of the aorta to the coronary sinus is believed to be threefold: (a) The myocardial capillary bed is perfused in retrograde fashion with arterial blood; (b) The myocardium is capable of extracting oxygen from the blood supplied to the myocardial capil-

lary bed in retrograde fashion; (c) There is a significant increase in the blood flow occurring *via* the intercoronary arterial anastomoses.

ACKNOWLEDGMENTS

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THE HEPATIC BLOOD FLOW AND SPLANCHNIC OXYGEN CONSUMPTION IN ALCOHOLIC FATTY LIVER

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Observations made during the development of choline-deficiency cirrhosis in animals have shown that fat accumulation precedes the appearance of fibrosis in the liver (1–3). Information obtained from autopsy and liver biopsy material (4–7) indicates that this probably also holds true for alcoholic cirrhosis in man. However, there is no agreement concerning the importance of the part which fat infiltration plays in the pathogenesis of either of these types of cirrhosis.

It has been suggested that compression of intralobular sinusoids by fat-distended liver cells impedes the intralobular circulation and that this, in part, contributes to the degeneration of centrilobular hepatic parenchyma (8). Evidence to support this hypothesis has been presented by Himsworth (8) and Hartroft (9), who studied sections of fatty livers from rats injected with India Ink prior to sacrifice. They demonstrated that in sections showing marked fat infiltration the penetration of sinusoids with ink was markedly limited as compared to the penetration of ink into the sinusoids of normal liver. In man, post-mortem hepatic perfusions carried out by Dock (10) showed a reduction in total hepatic perfusibility in large fatty livers.

The development of a method for estimating hepatic blood flow in man (11, 12) provided the means for further investigation into this problem. A direct comparison of the hepatic circulation of normal individuals and alcoholic patients with fatty livers was made possible. Furthermore, the rapid disappearance of fat from the livers of alcoholic patients following a period of therapy (7, 13) provided an opportunity to compare the hepatic blood flow in the same individuals before and after clearance of fat had occurred. The present paper reports the results of such a study.

MATERIAL AND METHODS

The hepatic blood flow was estimated in two groups of male subjects. The first consisted of 17 hospital patients who were considered "normal" inasmuch as they exhibited no evidence of cardiovascular, hepatic, biliary or gastrointestinal disease. The second group was made up of 12 alcoholic patients who were selected to include only men with fatty livers and without clinical signs of advanced cirrhosis. The following criteria were used for selection: 1) All admitted to a steady, daily, alcohol consumption, many months in duration, lasting up to the date of admission; 2) All gave histories of dietary deficiency; 3) Hepatomegaly was present in all cases; 4) None was jaundiced or febrile; 5) Splenomegaly or such evidence of portal hypertension as ascites or collateral veins was not demonstrable in any patient.

Each alcoholic patient was studied as soon after admission as practicable. The study was repeated, following a period of alcohol withdrawal, nutritious diet, and bed rest, when it was felt that the patient had achieved maximum improvement. At that time physical examination and the results of routine liver function studies no longer indicated the presence of liver disease. The time interval between the admission study and the study at "maximum improvement" of the alcoholic patients ranged from 21 to 116 days, the average interval being 44 days.

Essentially, the procedure described by Bradley, Ingelfinger, Bradley, and Curry (11) for estimating hepatic blood flow was followed. The patients were in the resting state, fasting, and had received Nembutal® 0.1 gm. one hour previously. A cardiac catheter was introduced into a medial antecubital vein and directed, under fluoroscopic vision, through the superior vena cava, right auricle, and inferior vena cava into a right hepatic vein. An infusion of bromsulfalein in isotonic saline (50 to 100 mgm. per 100 ml.) was started and a priming dose of approximately 2 mgm. per Kg. of bromsulfalein administered. A Graubard-Peterson Flowrator meter was used to maintain a constant infusion rate of 4 ml. per min. After an equilibration period of from 30 to 45 minutes, samples were drawn simultaneously from the hepatic vein and a peripheral vein or a brachial artery at intervals of from 7 to 10 minutes. Usually five pairs of specimens were thus obtained.

In addition to hepatic blood flow determinations in eight of the normal and eight of the alcoholic patients,

blood was drawn from the hepatic vein under anaerobic conditions for determination of oxygen content and calculation of splanchnic oxygen consumption. In these instances, when the collection of blood samples for the estimation of hepatic blood flow was completed, the catheter was withdrawn into the right auricle. Blood was then obtained simultaneously from the auricle and artery during the collection of a four-minute sample of expired air into a Douglas bag for calculation of cardiac output. Plasma volume was determined by the 10-minute Evans Blue Dye technique. Within 24 hours of the time of each hepatic vein catheterization, a needle biopsy of the liver was obtained.

When patients were studied twice, the second procedure duplicated the first in every respect. Every effort was made to replace the catheter in approximately the same area of the liver as in the first procedure.

Serum bromsulfalein levels were determined in duplicate with the Beckman spectrophotometer as described by Myers (12). Duplicate measurements exceeding 5 per cent in variation were discarded. All hemolyzed specimens were discarded. The concentration of bromsulfalein in infusion mixtures was determined similarly. Blood oxygen content was determined by the manometric method of Van Slyke and Neill (14). In the majority of instances duplicate detreminations checked within 0.10 ml. per 100 ml. and all checked within 0.15 ml. per 100 ml. Expired air volume was measured with a gasometer and the oxygen and carbon dioxide contents determined with the Scholander micro-gas analyzer (15).

Calculation of the hepatic blood flow with correction for variations in the peripheral bromsulfalein level was carried out according to Bradley, Ingelfinger, Bradley, and Curry (11). Where the rate of change of the peripheral bromsulfalein level exceeded .0002 mgm. per ml. per min. the experiment was discarded.

Liver biopsy specimens were fixed in 10 per cent formalin, sectioned, and stained with hematoxylin and eosin. The sections were examined and the results classified without knowledge of the patient's identity.

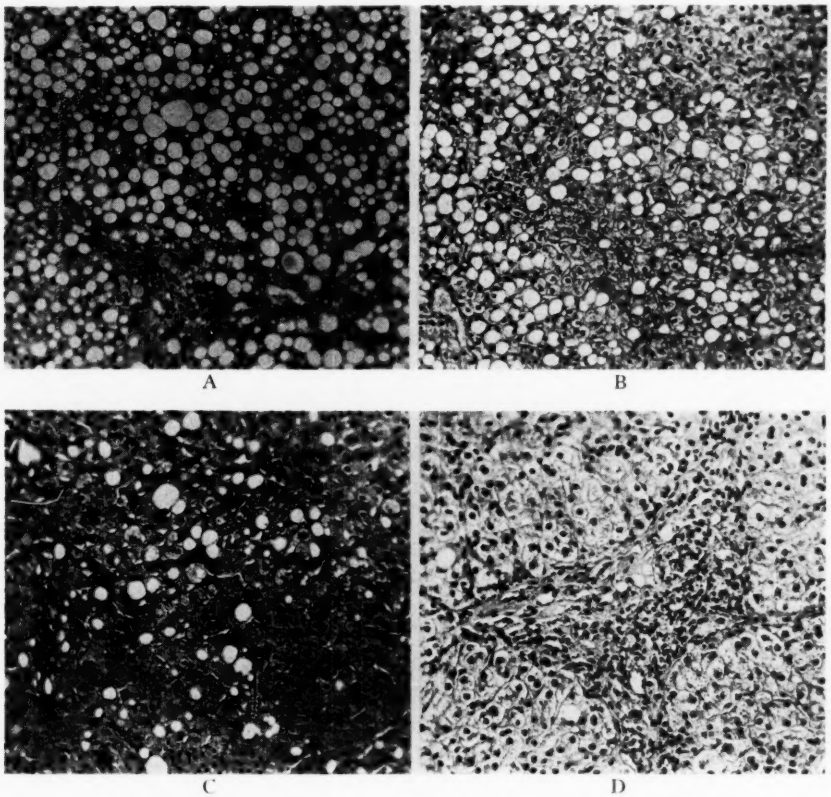

FIG. 1. LIVER BIOPSIES, SHOWING FATTY INFILTRATION OF MARKED (A), MODERATE (B) AND MILD DEGREE (C) AND INCREASED PERIPORTAL FIBROSIS (D)

TABLE I

Summary of hepatic blood flow observations on 17 normal and 12 alcoholic subjects

	EHBF* *ml./min./M²*			E(BSP)† *per cent*			C(BSP)‡ *ml./min./M²*		
	Range	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.
Normal subjects	550–1,120	770	154	33.7–82.0	56.9	12.9	187–306	241	38
Alcoholic patients No. 1§	430–1,090	650	170	15.9–72.8	44.3	19.6	44–253	154	63
Alcoholic patients No. 2‖	460–930	670	124	39.2–77.4	58.6	11.9	126–318	223	51

* Estimated hepatic blood flow.
† Hepatic bromsulfalein extraction.
‡ Hepatic bromsulfalein clearance.
§ Alcoholic patients studied shortly after admission.
‖ Same alcoholic patients studied after period of treatment.

RESULTS

Liver biopsies done in conjunction with the estimation of hepatic blood flow in alcoholic patients shortly after admission revealed fat infiltration of varying severity in all cases. In six cases it was marked, in four it was moderate, and in two it was minimal. Representative sections of these degrees of fat infiltration are illustrated in Figure 1. Biopsies obtained after treatment in the alcoholic patients showed varying degrees of fat clearance in all patients. At the time hepatic blood flow was restudied there was virtually complete disappearance of fat from the liver of nine patients and minimal fat infiltration remaining in three patients.

Increased periportal fibrosis was observed in the biopsies obtained from 9 of 12 alcoholic patients. In all these instances the fibrosis was minimal. Reduplication of bile ducts or architectural distortion of the liver was not seen. No fibrosis was found in any one of several biopsies done on each of the remaining three patients.

The data relating to hepatic blood flow are summarized in Table I. Although the mean estimated hepatic blood flow for the group of alcoholic patients shortly after admission is lower than that

TABLE II

Normal subjects

Subject	Age *yrs.*	Body surface area *M²*	Hematocrit *mm.*	P(BSP)* *mgm./100 ml.*	E(BSP)† *per cent*	C(BSP)‡ *ml./min./M²*	EHBF§ *ml./min./M²*
MAT	29	1.82	42.0	0.87	41.4	266	1.120
SAN	31	1.86	48.0	0.82	59.8	212	680
BUR	37	1.78	45.0	1.05	68.6	206	550
FOL	42	1.73	44.0	0.87	59.8	212	670
KLO	37	1.73	40.0	0.86	53.5	192	590
CLA	51	1.96	38.0	1.02	41.7	209	760
ORI	22	1.79	42.5	0.90	60.0	253	720
RUT	29	1.75	47.0	0.66	66.6	286	800
HUL	42	1.86	43.5	0.66	82.0	306	630
WAL	27	1.63	37.5	1.21	44.6	187	670
GRE	45	1.68	37.5	0.86	33.7	251	1,110
KEL	35	1.77	44.0	1.01	46.5	248	890
DUN	32	1.76	46.5	0.98	56.1	211	720
MEI	29	1.73	39.0	0.69	66.7	286	700
COH	25	1.81	44.0	0.77	66.4	294	800
SAL	27	1.86	42.0	0.65	73.8	203	750
BRO	38	1.61	37.0	0.77	46.8	275	880
Mean				0.86	56.9	241	770
S.D.				0.15	12.9	38	154
S.E.				0.04	3.1	4	37

* Peripheral bromsulfalein concentration.
† Bromsulfalein extraction.
‡ Bromsulfalein clearance.
§ Estimated hepatic blood flow.

TABLE III

Alcoholic subjects shortly after admission *

Subject	Age *yrs.*	Body surface area *M²*	Hematocrit *mm.*	P(BSP) *mgm./100 ml.*	E(BSP) *per cent*	C(BSP) *ml./min./M²*	EHBF *ml./min./M²*	Hepatic fat†	Hepatic fibrosis
COU	44	1.92	49.0	2.29	19.2	73	730	mar	X
PLE	40	1.82	40.0	2.76	16.3	44	470	mar	X
MLA	62	1.66	37.5	1.19	48.7	129	430	mar	0
GRE	35	1.67	38.0	0.72	51.4	192	610	mod	X
MOO	35	1.58	34.0	1.32	15.9	120	1,090	mar	X
DER	34	1.65	44.0	1.34	30.6	130	780	mod	0
COG	45	1.95	43.5	1.25	45.6	155	620	mar	X
DOW	46	1.82	44.0	0.55	67.3	253	670	mod	X
CIO	39	1.84	37.0	0.93	45.2	200	720	mod	X
MTI	52	2.02	52.0	1.34	45.5	113	500	mar	X
SCH	51	1.66	38.5	0.62	72.6	252	580	min	X
MIN	44	1.49	45.0	0.67	72.8	182	540	min	0
Mean				1.25	44.3	154	650		
S.D.				0.77	19.6	63	170		
S.E.				0.23	5.7	18	80		

* See Table II for explanation of abbreviations.
† mar—Marked fat infiltration.
mod—Moderate fat infiltration.
min—Minimal fat infiltration.

for the normal subjects (Tables II and III), the difference between the two means is not statistically significant (relative deviate, dev/S.E. = 1.9). When the hepatic blood flow in the alcoholic patients before and after the treatment period is compared (Tables III and IV), it may be seen that in three it decreased and in nine it increased. In six of the latter, however, the increase was less than 10 per cent. The mean hepatic blood flow after treatment was almost identical to that prior to treatment.

The mean hepatic extraction of bromsulfalein for the alcoholic group on admission was lower than that for the control group. The difference is not statistically significant (dev./S.E. = 1.9). Following the treatment period, the mean hepatic bromsulfalein extraction for the alcoholic patients was simliar to that for the control group. The difference between the mean bromsulfalein clearance for alcoholic patients, when first studied, and that for the control group is significant (dev./S.E. = 4.3). The mean bromsulfalein clearance for

TABLE IV

Alcoholic subjects after treatment period *

Subject	Body surface area *M²*	Hematocrit *mm.*	P(BSP) *mgm./100 ml.*	E(BSP) *per cent*	C(BSP) *ml./min./M²*	EHBF *ml./min./M²*	Hepatic fat	Hepatic fibrosis
COU	1.97	43.0	1.08	65.7	172	460	min.	X
PLE	1.82	44.5	1.38	45.7	126	500	min.	X
MLA	1.77	40.0	0.95	58.9	184	530	0	0
GRE	1.71	48.0	1.01	55.4	190	670	min.	X
MOO	1.66	38.5	0.74	39.2	230	930	0	X
DER	1.70	39.0	0.80	57.5	239	660	0	0
COG	1.98	46.0	0.81	69.1	236	630	0	X
DOW	1.87	42.5	0.63	73.0	299	730	0	X
CIO	1.86	39.5	0.90	43.3	214	790	0	X
MTI	1.98	47.0	0.73	68.5	257	710	0	X
SCH	1.75	40.5	0.96	49.0	210	720	0	X
MIN	1.59	40.0	0.53	77.4	318	700	0	0
Mean			0.88	58.6	223	670		
S.D.			0.31	11.9	51	124		
S.E.			0.09	3.4	15	36		

* See Table II for explanation of abbreviations.

TABLE V

Normal subjects

Subject	Oxygen consumption *ml./min./M²*	Cardiac index *L./min./M²*	Splanchnic oxygen consumption *ml./min./M²*	Splanchnic O_2 consumption as % of total O_2 consumption *per cent*	Hepatic vein oxygen content *ml./100 ml.*	Hepatic arterio-venous oxygen difference *ml./100 ml.*	Hepatic vein oxygen saturation *per cent*
WAL	116.7	3.3	32.5	27.8	10.7	4.9	62.1
GRE	158.6	4.4	32.9	20.7	11.3	3.4	71.0
KEL	151.5	3.9	26.9	17.8	16.7	3.0	77.9
DUN	134.0	3.6	21.8	16.3	16.0	3.0	77.4
MEI	146.3	4.3	21.0	14.4	13.4	3.0	71.8
COH	147.1	3.5	27.7	18.8	14.6	3.5	75.0
SAL	148.7	4.0	24.9	18.3	13.5	2.8	74.6
BRO	131.6	3.5	25.7	17.3	13.0	3.4	73.2
Mean	141.8	3.8	26.7	18.9		3.4	72.9
S.D.	12.8	0.4	4.3	3.8		0.6	4.8
S.E.	4.5	0.1	1.5	1.3		0.2	1.7

Alcoholic patients shortly after admission

Subject	Oxygen consumption *ml./min./M²*	Cardiac index *L./min./M²*	Splanchnic oxygen consumption *ml./min./M²*	Splanchnic O_2 consumption as % of total O_2 consumption *per cent*	Hepatic vein oxygen content *ml./100 ml.*	Hepatic arterio-venous oxygen difference *ml./100 ml.*	Hepatic vein oxygen saturation *per cent*	Hospital day test performed
MOO			75.2		9.0	6.9	58.4	5
DER	171.1	3.6	62.6	36.6	8.4	8.0	41.0	1
COG	145.3	3.3	38.1	26.2	11.9	6.1	63.9	4
DOW	177.9	2.9	61.6	34.6	8.1	9.2	44.5	2
CIO	167.8	3.6	38.2	22.8	10.4	5.3	61.1	6
MTI	168.1	2.8	32.7	19.5	12.4	6.5	57.1	4
SCH	170.8	4.2	36.8	21.5	7.8	6.3	48.7	1
MIN	174.0	3.6	35.8	20.6	10.2	6.6	52.2	2
Mean	167.9	3.4	47.6	26.0		6.9	53.4	
S.D.	9.8	0.4	15.2	6.4		1.2	7.6	
S.E.	3.7	0.2	5.5	2.4		0.4	2.7	

Alcoholic patients after treatment

MOO			40.9		11.7	4.4	67.8	37
DER	164.3	4.2	25.5	15.5	11.6	3.9	67.4	29
COG	122.5	2.9	25.5	20.8	14.3	4.1	74.9	36
DOW	183.6	5.3	34.3	18.7	12.0	4.7	65.9	50
CIO	158.5	3.9	32.3	20.4	11.3	4.1	67.7	50
MTI	119.5	3.6	32.0	26.8	12.5	4.5	60.8	120
SCH	146.3	4.7	26.0	17.8	11.3	3.6	69.9	55
MIN	144.3	3.8	26.1	18.0	13.7	3.7	74.8	38
Mean	148.4	4.1	30.3	19.7		4.1	68.6	
S.D.	21.1	0.7	5.1	3.3		0.4	4.4	
S.E.	8.0	0.3	1.8	1.3		0.1	1.5	

alcoholic patients after the treatment period was not significantly different from the control mean (dev./S.E. = 1.0).

Table V contains the above-mentioned, additional data on eight normal and eight alcoholic subjects. The calculated oxygen uptake in the splanchnic area in the normal subjects ranged from 21.0 to 32.9 ml. per min. per sq. M., the mean being 26.7 ± 4.3 ml. In the alcoholic patients, when first studied, the splanchnic oxygen uptake ranged from 32.7 to 75.2 ml. per min. per sq. M., the mean being 47.6 ± 15.2 ml. Following treatment, the splanchnic oxygen uptake for these alcoholic patients ranged from 25.5 to 40.9 ml. per min. per sq. M., the mean (30.3 ± 5.1 ml.) having returned to a level only slightly higher than the control mean.

The average total oxygen consumption in the alcoholic patients before treatment was 167.9 ±

9.8 ml. per sq. M. per min., which was higher than the average in the same patients following treatment (148.4 ± 21.1 ml. per sq. M. per min.). The difference in these averages is 19.5 ml., which matches closely the difference in averages of the splanchnic oxygen consumption before and after treatment Th splanchnic oxygn consumption, when expressed as a percentage of the corresponding total oxygen consumption, was 27 per cent before treatment and 20 per cent after treatment. In the normal individuals the splanchnic oxygen consumption comprised 19 per cent of the total oxygen consumption.

The mean hepatic arterio-venous oxygen difference and mean hepatic venous oxygen saturation for the normal group were 3.4 ± 0.6 ml. per 100 ml. and 72.9 ± 4.8 per cent, respectively. In the alcoholic patients, the average hepatic arterio-venous oxygen difference was 6.9 ± 1.2 ml. per 100 ml., shortly after admission and 4.1 ± 0.4 ml. per 100 ml., after the treatment period. The average hepatic venous oxygen saturation in the alcoholic individuals was 53.4 ± 7.6 per cent before treatment and 68.7 ± 4.4 per cent after treatment.

DISCUSSION

The results of hepatic blood flow measurements indicate that fat infiltration in the liver of alcoholic patients does not result in an impairment of the total hepatic blood circulation. The fact that in the group of alcoholic patients, before and after fat disappearance, the mean hepatic blood flow was somewhat lower than normal is not surprising inasmuch as the liver biopsies of the majority of these patients showed increased fibrosis. Bradley, Ingelfinger, and Bradley (16) reported a significant decrease in the hepatic blood flow of individuals with advanced cirrhosis. Their patients exhibited clinical evidence of portal hypertension and an average hepatic bromsulfalein extraction significantly lower than normal. The alcoholic patients in the present study differed from those of Bradley, Inglefinger, and Bradley. Following a period of treatment, they displayed no clinical signs of liver disease and their averages for bromsulfalein extraction and bromsulfalein clearance were quite similar to the normal group. These findings confirm the impression, obtained histologicaly, that the cirrhosis was minimal if present. This may explain the lack of a statistically significant difference in mean hepatic blood flow between the normal and alcoholic subjects although it is possible that if these studies were extended to larger groups this difference might then become significant.

The increased splanchnic oxygen uptake in the alcoholic patients shortly after admission is of considerable interest. Although it is not possible to determine whether or not this increase in oxygen consumption occurred in the liver, there is no reason to account for it elsewhere in the splanchnic area. Splanchnic oxygen consumption is the calculated product of splanchnic blood flow and the hepatic arterio-venous oxygen difference. Since, in these studies, the average splanchnic blood flow was lower than normal, the increase in splanchnic oxygen consumption was accounted for solely in the hepatic arterio-venous difference. The average value for this difference was 65 per cent greater in the initial test of the alcoholic patients than in the test following the treatment period and 100 per cent greater than the average value for the normal subjects.

The reduction which occurred in the hepatic arterio-venous oxygen difference following the treatment period was more striking in some patients than in others. This did not appear to be related to the severity of fat infiltration, but rather to the time interval between admission and the initial hepatic vein catheterization. Thus, the greatest percentage change in hepatic arterio-venous oxygen difference occurred in the four patients whose initial test was done on the first and second days after admission. In these patients the initial liver biopsy showed moderate fatty infiltration in two and mild fatty infiltration in two. The least percentage change in hepatic arterio-venous oxygen difference occurred in the four patients whose initial study was done on the fourth, fifth, and sixth days after admission. Yet the initial liver biopsies in these patients showed fatty infiltration which was moderate in one and marked in three. This was also true for the values of hepatic venous oxygen saturation. The patients studied on the first and second days after admission showed the lowest saturations. Because none of the alcoholic patients was seriously intoxicated at the time of admission, it seems unlikely that these findings

are related to alcohol oxidation in the liver 24 to 48 hours after alcohol withdrawal. Moreover, elevated splanchnic oxygen uptakes four to six days after the day of alcohol withdrawal makes this possibility even less likely.

It would appear, therefore, that in the fatty livers of alcoholic patients, as a result of increased splanchnic oxygen uptake and subnormal hepatic blood flow, the centrolobular hepatic cells are perfused by blood with oxygen tensions which are lower than normal. Myers and Hickam have shown that centrolobular anoxia exists in heart failure (17) and thyrotoxicosis (18) and they have considered the possible relation of this finding to the centrolobular necrosis which may be found in either of these diseases. Centrolobular necrosis is not a prominent feature in the histological picture of alcoholic fatty liver. However, the indication of existing centrolobular anoxia in this disease in part supports the hypothesis advanced by Himsworth (8) that anoxia produces slow atrophy of centrolobular cells and the eventual development of hepatic fibrosis.

SUMMARY

1. The presence of fatty infiltration of the liver in alcoholic patients does not appear to alter hepatic blood flow as estimated by the bromsulfalein technique. In 12 such patients with histologically demonstrated fatty livers, the mean hepatic blood flow was slightly, although not significantly, lower than that for 17 normal individuals. Moreover, after histological demonstration of fat disappearance in the same alcoholic patients, the mean hepatic blood flow was the same.

2. In eight of the alcoholic patients and eight of the normal individuals, the hepatic arterio-venous oxygen difference was determined. In the alcoholic patients prior to treatment, increases in the splanchnic oxygen consumption and hepatic arterio-venous oxygen difference were found. These were associated with lowered values for hepatic venous oxygen saturation. The possibility that this anoxia is of significance as a contributing factor to the damage of centrolobular hepatic cells is raised.

3. Before treatment the alcoholic patients showed a decreased ability to excrete bromsulfalein as evidenced by lowered bromsulfalein extraction and bromsulfalein clearance. These values returned to normal following the treatment period. There was a corresponding return of the hepatic arterio-venous oxygen difference to near normal values.

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EFFECTS OF INFUSION OF HYPERONCOTIC DEXTRAN IN CHILDREN WITH THE NEPHROTIC SYNDROME [1]

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Dextran is a bacterial polysaccharide which is being increasingly used as a plasma substitute (1–4). Dextran molecules frequently have a molecular weight of several million, and consist of long branched chains of glucose units. For clinical use these large molecules are broken down artificially to an average molecular weight of 70,000. In the body, the larger molecular aggregates are further broken down and excreted as smaller fractions having (approximate) molecular weights less than 20,000, or are slowly metabolized.

Dextran has been reported to induce diuresis regularly in patients with the nephrotic syndrome (5, 6). In addition to determining the clinical value of dextran, the intravenous infusion of hyperoncotic (12 per cent) dextran in water was used as an approach to the study of the following questions:

1) Effect of increased plasma volume on glomerular filtration rate;

2) The relation of plasma volume, serum albumin concentration, and glomerular filtration rate to the glomerular permeability to albumin;

3) The effect of increased post-glomerular colloid osmotic pressure on tubular reabsorption of water and solutes;

4) The nature of the dextran-induced diuresis.

METHOD

Nephrotic children who showed no clinical evidence of renal failure were selected (*i.e.*, no concomitant acidosis, azotemia and anemia). The children were recumbent during the study period. Infusions were administered in the morning after an overnight fast; during the fasting period water was given when requested by the children, but no deliberate attempt was made to pre-hydrate them. A 12 per cent solution of dextran in distilled water [4] was used, 300 to 400 ml. per M^2 (1.2 to 1.8 gm. dextran per Kg. body weight) given by infusion daily or on alternate days at a rate of 2 to 4 ml. per minute. The blood pressure was checked at 20 minute intervals and if the systolic pressure reached 140 mm. Hg, infusion was stopped.

Plasma volume was measured by modification of the method of Chinard and Eder (7) from dilution of T-1824 measured at 10, 20, and 30 minutes after injection. Where satisfactory extrapolation curves were not obtained in serial measurements, calculated values are presented, assuming an initial average plasma volume of 1.2 L. per M^2 (8). The change in plasma volume with dextran infusion was estimated. Since hemoglobin was not measured, the formula $PV_2 = PV_1\left(\frac{1 - Hct_2}{1 - Hct_1}\right)^2$ was used. This empirical formula is roughly comparable to the usual $\frac{PV_2}{PV_1} = \frac{Hb_1}{Hb_2} \times \frac{1 - Hct_2}{1 - Hct_1}$. Hematocrits were determined by the usual Wintrobe method (9) and were not corrected for trapped plasma. In calculating the infusion volume required and in the data presented in the tables, estimates of surface area were based on height and presumed (age-height) or observed non-edematous weight. Total solute concentrations in serum and urine were measured by freezing-point depression using a Thermistor 1B resistance probe and a wheatstone bridge osmometer.[5] Serum and urine dextran levels were measured by the methods of Bloom and Willcox (10) and Metcalf and Rousselot (11). Since endogenous creatinine clearance may be an unreliable measure of glomerular filtration rate (GFR) in the presence of renal disease (12) and dextran interferes with the standard Roe method of de-

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[4] Batches No. 263 H7 and 278 P7, kindly prepared for us and supplied with the following comments by Commercial Solvents Inc., Terre Haute, Indiana. About 80 per cent of the polysaccharides in these batches had an average molecular weight of 70,000. About 10 per cent had a low molecular weight of 30,000; 10 per cent had a high molecular weight of 180,000.

[5] Made by Fiske Associates, Boston, Massachusetts.

termining inulin,[6] glomerular filtration rate was measured by continuous infusion (rather than a single injection [14]) of sodium thiosulfate in four patients although this substance is known to produce a solute diuresis. Effective renal plasma flow (RPF) was measured by p-aminohippurate in these four and an additional three patients. In these patients the urines were collected by catheter and the bladder emptied with distilled water and air rinses in the usual fashion. Albumin in serum and urine was determined immunochemically (15) in four patients. Sodium and potassium in serum and urine were determined by an internal standard flame photometer (16), and chlorides in urine were measured by the method of Wilson and Ball (17).

[6] Quantitative methods for the simultaneous determination of separate sugars in biological fluids are available (13), but to date have not given consistent results in our hands.

RESULTS

The clinical efficacy of hyperoncotic dextran as a diuretic agent in the nephrotic syndrome is indicated in Table I. Primary and derived physiologic data are presented in Tables II–IV, and Figure 1. A complete protocol of the course of diuresis in one patient is given in Table V, and estimates of glomerular permeability to albumin and water in Table VI. The effect of pitressin on dextran-induced diuresis is shown in Figure 2.

TABLE I

Clinical results of dextran infusions

Patient	Age *yrs.*	Number of infusions*	Wt. before therapy *Kg.*	Least wt. after therapy *Kg.*	Least wt. days after last infusion	Wt. loss *Kg.*	Estimated non-edema weight *Kg.*	Proportion of edematous wt. lost %
S. L. B.	11 6/12	4	60.0	46.0	1	14.0	46.0	100
S. L. B.	11 10/12	8	57.5	47 7	1	9.8	46.0	85
M. N.	3 1/12	5	18.5	14.5	2	4.0	14.0	88
W. D.	9 3/12	4	43.3	30.8	3	12.5	27.0	77
S. W.	6 7/12	4	27.0	21.4	2	4.3	21.0	93
J. R.	3 0/12	3	16.2	13.9	2	2.3	12.0	55
J. F.	3 6/12	3	18.1	17.1	6	1.0	14.5	27
N. McC.	4 1/12	5	25.7	22.7	1	3.0	20.0	54
C. B.	2 9/12	5	20.0	15.9	2	4.1	14.5	74
C. B.	2 11/12	4	21.6	15.1	2	6.5	14.5	92
B. L.	3 0/12	5	24.7	13.0†	8	11.7	13.0	100
B. L.	3 1/12	3	18.2	18.7	—	—	13.0	0
D. G.	4 11/12	4	19.5	16.5‡	1	3.0	15.0	67
C. F.	6 8/12	4	23.1	22.1	1	1.0	19.0	24
J. G.	4 8/12	3	19.4	18.9	1	0.5	14.0	10
P. M.	6 3/12	3	28.4	27.5	1	0.9	18.0	9

* Each infusion on separate, usually consecutive, days.

† Also receiving ephedrine sulfate for asthma during this period. Ephedrine sulfate, p.o., may produce diuresis in occasional patients with the nephrotic syndrome (18).

‡ Slight paracentesis drainage persisted on first two days of infusions.

TABLE II

Effect of dextran on plasma volume—all data adjusted to 1 Sq.M. S.A.

Patient	Intravenous dextran *gm.*	HCT % Initial	HCT % Final	Pl. vol.* *ml.* Initial	Pl. vol.* *ml.* Final	%Δ	ΔPV per gm. infused dextran‡ *ml.*
D. G.	43.5	26	14	1,240	1,675	+35	10.0
S. B. (1)	32.6	42	35	1,360	1,710	+26	10.7
(2)	33.4	45	36	1,910	2,580	+35	19.8
C. B.	49.1	35	25	1,200†	1,600	+33	8.1
M. N. (1)	30.0	40	28	1,320	1,900	+44	19.3
(2)	49.0	34	23	1,980	2,690	+36	20.9
J. L.	37.3	35	21	1,200†	1,770	+48	15.3

* Initial plasma volume determined by T-1824 dilution. Final plasma volume calculated from change in hematocrit.

† Not measured but estimated as normal volume.

‡ Plasma volume increments would be higher if quantity retained was used, rather than quantity infused. These data were available only in S. B. (2), C. B., and M. N. (1).

TABLE III

Effect of plasma volume expansion on total circulating albumin

Patient	Δ plasma volume %	Total circulating albumin		Excreted albumin gm.	Albumin from "Extravascular Pool" gm.
		Initial gm.	Final gm.		
D. G.	+35	1.4	1.3	0.5	0.4
S. B. (1)	+26	10.3	7.9	1.9	—
(2)	+35	27.5	27.4	0.7	0.6
M. N. (1)	+44	2.2	2.2	0.5	0.5

Clinical effects (Table I)

Courses consisting of three or more daily infusions have now been given to thirteen children and three children have received two or more such courses. A significant loss of edema occurred in nine children, and diuresis was virtually complete in six. Least body weight was usually attained within 24 to 48 hours after the last infusion. Of the remaining four children, two (J. G. and P. M.) were known to have reduced values of inulin and PAH clearances and have since died of renal failure. Figure 1 depicts some features of a successful course of treatment.

The majority of children developed mild hypertension (20 mm. + elevation of systolic and occasionaly diastolic pressure),[7] during the infusions, associated in some instances with headache, nausea and abdominal pain. These symptoms subsided within 24 hours in all instances. Infiltration of dextran into the subcutaneous tissues in

[7] If hypertension exceeded 140/100 mm. Hg, infusions were discontinued.

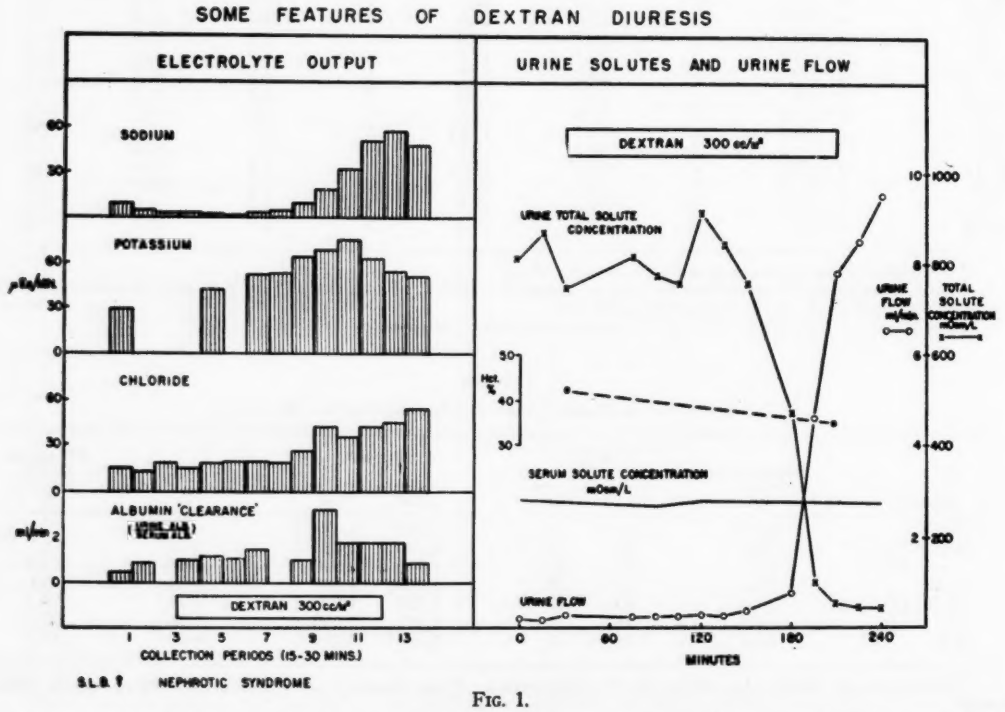

FIG. 1.

Patient S. B.(1) received 380 ml. of 12 per cent dextran in water. Renal functions were within normal limits immediately prior to infusion (Table IV). Water diuresis preceded the increased excretion of solute.

TABLE IV

Average values for three consecutive periods before dextran infusion and during maximal dextran diuresis

Patient	Sex	Age yrs.	S.A. M^2	Date	Infusion 12% dextran ml.	Period numbers (incl.)	minutes	Rates of excretion in urine/M^2/min.								Serum concentrations					Renal clearance			
								H_2O	Na	K	Cl	S_2O_3	Total solutes		Alb.	Dextran	Na	K	T.S.	Alb.	Dextran	GFR	RPF	FF	C Alb.
								$\mu Eq.$					$\mu Osm.$	$mOsm./L.$	$mg.$		$mEq./L.$		$mOsm./L.$	$mg./ml.$		$ml./min./M^2$			
D. G.	M	4 11/12	0.62	12/11/52	225	2–4	33–79	0.4	7	41	9	—	234	492	6.0	—	131	3.6	280	1.8	—	25*	266	0.10	3.8
						11–13	201–246	10.3	266	64	232	—	774	75	4.3	—	129	3.1	279	1.2	—	—	356	—	4.2
S. B. (1)	F	11 6/12	1.40	12/30/52	380	2–4	30–75	0.2	16	25	22	—	165	806	3.5	—	153	4.2	293	5.4	—	57*	331	0.17	0.6
						18–20	315–360	6.2	37	40	34	—	292	47	6.7	—	144	3.7	275	3.3	—	—	464	—	1.1
(2)		11 10/12		4/17/53	390	2–4	30–75	1.1	241	107	5	241	681	618	1.5	—	—	—	275	10.6	—	91	208	0.44	0.1
						9–11	180–225	6.6	261	85	18	109	672	103	4.4	98	—	—	258	7.6	18.7	122	404	0.30	0.6
C. B.	F	2 7/12	0.55	3/13/53	225	2–4	30–75	0.4	4	49	2	—	276	783	—	—	140	4.9	273	—	—	27*	317	0.09	—
						9–11	167–212	3.5	30	67	37	—	403	118	—	47	126	3.8	271	—	19.2	—	450	—	—
M. N. (1)	F	3 1/12	0.60	5/1/53	150	2–4	30–75	1.0	87	403	—	208	598	619	3.1	—	156	2.2	290	2.8	—	49	185	0.25	1.1
						8–10	175–220	10.5	392	163	—	216	889	84	9.0	56	145	1.0	274	1.9	14.4	72	402	0.18	4.8
(2)				5/29/53	245	2–4	30–75	1.2	102	115	15	193	461	390	—	—	141	4.6	287	—	—	68	318	0.21	—
						7–9	135–180	7.1	200	136	116	218	640	92	—	—	139	3.0	268	—	—	67	524	0.13	—
J. L.	M	1 7/12	0.45	6/9/53	140	2–4	30–75	1.2	81	207	21	191	538	455	—	—	140	1.6	268	—	—	61	408	0.15	—
						6–8	105–160	7.3	182	239	108	162	746	138	—	—	140	0.5	257	—	—	69	615	0.11	—

* Inulin. Other GFR's determined with $Na_2S_2O_3$.

TABLE V

Protocol: M. N. (I) 3 1/12 yrs. SA = 0.60 W = 18.9 Kg. H = 97 cm.; 5/1/53
Simultaneous thiosulfate and p-Aminohippurate clearances, 150 ml. dextran from 75 to 235 min.

Time min.	Urine excretion/min./M²									Serum						Renal clearance				Plasma volume	
	H_2O ml.	Na μEq.	K μEq.	Cl μEq.	Thio μEq.	Total μOsm.	Solutes mOsm./L.	Alb. mg.	Dextran mg.	Na	K	Thio	T.S. mOsm./L.	Dextran	Alb.	Alb.	GFR	RPF	FF	HCT %	PV_{T1824} ml.
										mEq./L.				mg./ml.		ml./min./M²					
−68–0	0.2	1	60	71		182	910			156	2.2		290		2.9					40	794
0–30	1.2*	112	430	62	228	724	604							0.02							
45	1.0	100	422	7	232	624	624	3.2				5.7			2.9	1.1	40	133			
60	1.0	88	424	7	207	624	628	3.5				3.8				1.2	55	200			
75	0.9	72	363	3	191	544	604	2.7	0.3			3.6			2.7	1.0	53	222			
75–115	0.9	87	395	5	207	342	380	2.2	17.7			3.6				0.9	57	334	0.17		
145	1.0	83	387	4	195	562	562	3.6	33.4			3.6		14.9	2.2	1.6	53	388	0.14		
175	3.3	118	526	27	243	740	224	3.9	69.2			3.1	268		2.1	1.9	77	516	0.15		
190	8.3	225	205	28	226	697	84	6.7	91.8			3.0				3.4	77	378	0.20	28	
205	14.3	489	148	—	236	930	65	10.9	88.0			3.0	274			6.0	80	462	0.17		
220	10.0	463	135	282	190	1,030	103	9.4	68.0			3.2				5.5	60	367	0.16		
235	4.8	447	132	259	211	1,186	247		59.2	145	1.1	3.4		13.9	1.6		63	400	0.16	28	1,145
265	3.7	374	107	87		926	250		42.2												
295	1.5	168	95	50		485	323		30												
325	1.2	142	97	43		492	410		32												
355	0.9	112	90	30		423	470		48												

* The increased excretions of water and solutes, particularly sodium and potassium, during the pre-dextran periods resulted from sodium thiosulfate infusion.

TABLE VI

Hypothetical estimates of relative "filtration" of albumin compared with that of water during dextran diuresis (M. N. 5/1/53)

Time *min.*	Serum albumin *mg./ml.*	Filtered/min. H_2O *ml.*	Filtered/min. Albumin* A *mg.*	B *mg./100 ml.*	C *mg./100 ml.*	$\frac{C}{B}$	Excreted/min. H_2O *ml.*	Excreted/min. Albumin *mg.*	"C Alb" *ml./min.*
C	2.9						0.12	—	—
0–30							0.70	—	—
30–45	(2.9)†	24	70	1.9	7.9	4	0.60	1.89	0.65
45–60	2.9	33	96	1.9	6.3	3	0.60	2.08	0.72
60–75	2.7	32	87	1.8	4.9	3	0.56	1.58	0.58
75–115	(2.5)	34	85	1.7	3.9	2	0.54	1.34	0.54
115–145	2.2	32	70	1.5	6.8	5	0.62	2.18	0.95
145–175	2.1	46	97	1.4	5.1	3	2.0	2.33	1.1
175–190	(2.0)	46	92	1.3	8.7	7	5.0	4.0	1.97
190–205	(1.9)	48	91	1.3	13.6	10	8.6	6.52	3.5
205–220	(1.8)	36	65	1.2	15.7	11	6.0	5.66	3.2
220–235	1.6	38	61	1.1	—		2.9	—	—

* Albumin

$A = GFR_{H_2O} \times$ serum albumin (mg./ml.) = "maximal" filtered albumin (mg./min.).

$B = 100 \times \left(\frac{0.020}{3.0} \times \text{serum albumin (mg./ml.)} \right)$ = "proportional" filtered albumin/100 ml. glomerular filtrate.

$C = \frac{\text{urine albumin (mg./min.)}}{GFR_{H_2O}} \times 100$ = "minimal" filtered albumin/100 ml. glomerular filtrate.

$\frac{C}{B}$ = ratio of "minimal" to "proportional" filtered albumin; *i.e.*, estimate of glomerular permeability to a constant fraction of serum albumin.

Note that neither A nor B have physiologic validity (see Discussion).

† Figures in ( ) interpolated from semilog plot. Periods 0–75 minutes before dextran; 75–235 minutes, during dextran.

three instances led to a swollen painful arm for several days. Several children had post-infusion epistaxis.[8] Possible adverse effects of repeated infusions are undetermined.

Edema recurred rapidly in all patients, the longest period of remission following therapy was one month.

Physiological effects

Plasma volume: The hyperoncotic dextran infusions regularly induced increases of 25 to 50 per cent in plasma volume. The oncotic effect of dextran in acutely expanding plasma volume varied, but averaged about 15 ml. per gm. dextran infused per square meter surface area (Table II) by the end of the infusion. Satisfactory measurements of urinary dextran excretion were made in two patients (S. B.$_2$ and M. N.$_1$). These indicated that 29 and 43 per cent (respectively) of the infused dextran was excreted during the period of infusion; and if the oncotic effect was calculated from the amount retained at the end of infusion rather than the total quantity infused, higher values were obtained (28 and 34 ml. per gm. rather than 20 and 19 ml.). This calculation can only be an approximation since the final estimated value for total circulating plus excreted dextran in M. N. was slightly greater than the amount actually infused, also the estimates of final plasma volume increments from fall in hematocrit may be inaccurate.

The increment of plasma volume was associated with a decreased concentration of serum albumin; however, the total circulating albumin decreased in only one of four instances in which it was measured (Table III). If the amount of albumin excreted in the urine during dextran infusion was considered, then the maintenance of total circulating albumin during acute episodes of depletion seems to have been at the expense of the "extravascular pool." Calculations of this type based on crude estimates of plasmas volume must be interpreted cautiously.

[8] Since this report was prepared, two children have developed hematuria during the 2nd or 3rd dextran infusion. This cleared spontaneously in 4 to 7 days.

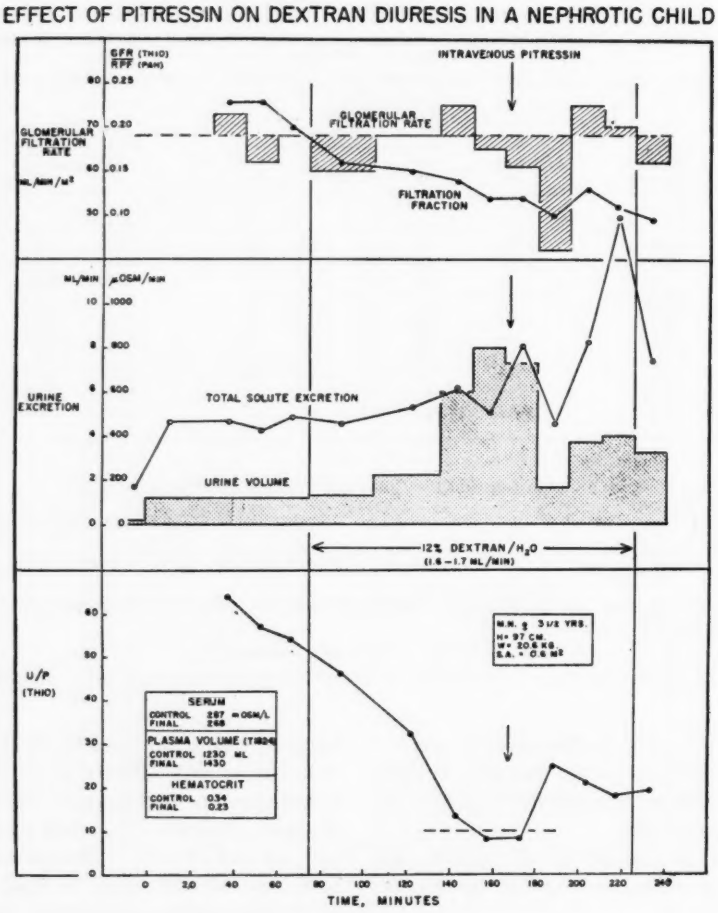

FIG. 2.

Patient M. N.(2). Note reduction in filtration fraction, and water diuresis preceding solute diuresis prior to pitressin injection. The pitressin response indicates that the renal tubules were responsive to increased antidiuretic hormone in the presence of high levels of circulating dextran.

Serum solutes: Serum total osmolarity and electrolyte concentrations decreased during dextran infusion, typified by examples cited in Table IV.

Diuresis: Averaged data for three consecutive control periods and three consecutive periods during maximal diuresis obtained during seven studies on five patients are noted in Table IV.

Urine flow during the control periods was usually low. No change was noted until the infusion had been running for about 1½ to 2 hours, when a sharp increase in urine flow occurred, output often increasing 10-fold within 30 minutes (Figure 1). The urinary total solute output increased in all but one instance, although total solute concentration fell to a level much below that in the plasma, indicating a water diuresis.

The protocol of M. N. (Table V) indicates that the peak excretion of dextran more or less coincides with that of water, and both precede the periods of maximum total solute excretion. The

terminal rise in dextran excretion may be insignificant.

Sodium and chloride excretion increased during the later phases of diuresis in all cases, often to a striking degree. Usually, maximal water diuresis preceded maximal sodium output. There were insignificant fluctuations in potassium excretion.

In one child with persistent edema (C. B.),[9] dextran infusion failed to produce significant diuresis in spite of adequate infusion volume and a marked fall in hematocrit. Profuse diuresis had been induced two days, and two months previously. Sodium excretion during the control period was relatively high, and increased slightly during the infusion. There was no significant change in urine solute concentration.

In most cases urine flow and electrolyte output returned to control levels during the several hours following infusions. In a few patients, however, increased urine flow persisted accompanied by continued excretion of sodium and chloride.

Glomerular filtration rate (C Thio) was measured satisfactorily during dextran infusion in four cases, and increased significantly in two. It did not change significantly in the other instances despite significant increases of plasma volume. Renal plasma flow (C PAH) was increased markedly in all seven cases in which it was determined. Owing to the proportionately greater increase in p-amino-hippurate clearance, filtration fraction fell in all instances where estimated.

Effect of pitressin on diuresis

In two children, (J. L. and M. N.), pitressin (2 units per M^2) was given intravenously after diuresis had become established. Injection was associated with nausea, retching and pallor. After a latent period of about ten minutes, urine flow decreased to levels similar to those seen in the control periods (Figure 2). Electrolyte excretion fell simultaneously but to a lesser extent so that urinary total solute concentration rose. Glomerular filtration rate and renal plasma flow were also transiently reduced. The filtration fraction was not definitely altered. With the exception of urine flow, which increased only slightly, after about 30 minutes all measurements returned toward their previous diuretic levels.

Albumin excretion

Serum dextran levels rose to about 2.0 gm. per 100 ml. during the infusions. (Maximal urinary dextran excretion rates occurred prior to the onset of diuresis, due probably to the early excretion of smaller molecules.) Measurements of serum and urine albumin concentrations were made in four patients.[10] Although total circulating albumin was not usually changed (Table III), serum albumin concentration consistently fell during dextran infusion (Table IV). Urine albumin excretion increased in three of the four patients in which it was measured and fell in one. Albumin "clearance" (C Alb) increased in three of the four patients. In two of these, the filtration of water was measured and also appeared to be increased.

DISCUSSION

The present findings confirm previous reports (5, 6) that dextran infusions induce a temporary diuresis in the nephrotic syndrome. Dextran therapy may prove to be of value when it is desirable to reduce edema prior to other therapeutic procedures, and measures, ordinarily used to reduce edema, are either impracticable or contraindicated. Contraindications to dextran therapy are pre-existing hypertension, which may be aggravated, and massive anasarca, when a rapid increase in plasma volume may further embarrass the cardiovascular system. Some data are available suggesting that dextran may have a hemolytic effect (19). In general better diureses were obtained when a greater number of infusions were given. From present experience it appears that at least three to five consecutive infusions should be given to obtain maximum benefit.

A marked increase in plasma volume was produced, and in the patients studied this was associated with rise in renal plasma flow. Glomerular-filtration rate, however, rose in only two of four patients in whom observations were made. The general effects appear to resemble those produced by infusion of hyperoncotic salt-poor human serum albumin (20, 21), except that in the patients stud-

[9] This study, and two others, were not included in Table IV since renal clearance data were not obtained.

[10] We are indebted to Dr. Dominick Conway for these measurements.

ied by Orloff, Welt, and Stowe the solute concentration (sodium) in the extracellular fluid increased during infusion.

In previous studies to determine the effect of dextran on renal function, a 6 per cent solution of dextran was used, producing an average drop of 12 per cent in hematocrit and slight increases, thought to be insignificant, in renal function in normal subjects (22). Increases in both glomerular filtration rate and renal plasma flow have been noted in animals (23). In the present study, a 12 per cent solution was used, and the average fall in hematocrit was 30 per cent indicating that a larger increase in plasma volume was achieved, due perhaps to the hyperoncotic solution used and the existing hypoproteinemic state (21). This larger increase in plasma volume may account for the increases in renal plasma flow observed in the present study. The increased plasma volume may be the stimulus to diuresis by increasing glomerular filtration rate as previously suggested (20, 21, 24); however, the data indicate that increased plasma volume and systemic hypertension both favoring increased filtration pressure do not necessarily increase the glomerular filtration rate. It is possible that the combined counteracting colloid osmotic effects of dextran plus albumin were approximately equal to the rise in filtration pressure or that the basement membranes were sufficiently altered by disease to make acute changes in filtration unlikely. Therefore, in the patients in whom glomerular filtration rate did not increase, dextran diuresis was clearly a renal tubular phenomenon. When glomerular filtration rate was increased, it would seem that either rise in filtration pressure exceeded the counteracting colloid osmotic effect and/or an increased number of glomeruli were being perfused. One might speculate, for instance, if severe interstitial renal edema caused partial extrinsic occlusion of some afferent glomerular arterioles, the rise in mean blood pressure might be sufficient both to improve individual glomerular perfusion and to increase the total number of glomeruli perfused. Increased colloid osmotic pressure in the post-glomerular blood flow might reduce intrarenal peritubular edema.

The apparent relationship between filtration of water and albumin is largely dependent upon how the amount of albumin filtered is estimated. This is indicated by several different methods of handling the same data obtained in M. $N._1$ (Table VI). Three possible series of values for filtered albumin are shown in the columns headed A, B, and C. "A" defines a glomerular clearance situation in which all serum albumin is filtered. This is obviously a maximal limiting value and is physiologically unlikely. "B" defines the situation in which some constant proportion of serum albumin is filtered, the actual amount being dependent upon the level of serum protein and the glomerular filtration rate. The constant 0.020/3.0 was chosen since it has been assumed on the basis of studies in rats and guinea pigs (25) that with normal levels of serum albumin (3.0 gm. per 100 ml.), 0.020 gm. are filtered per 100 ml. of filtrate formed (26). Estimated in this way, the concentration of albumin per 100 ml. of glomerular filtrate appears to *decrease* with dextran diuresis. Actually, this result need not imply any change in permeability to albumin but depends upon the fact that the decrease in concentration (dilution) of serum albumin was proportionately greater than the increase in filtered water. This is particularly evident upon comparison of the periods from 45 to 115 minutes with those of 205 to 235 minutes. However, the arbitrary ratio 0.020/3.0 × serum albumin (mg. per ml.) × GFR yields values for albumin filtered per minute which are less than amounts appearing in the urine per minute in the respective periods. If the average observed ratio of albumin recovered in the urine to the "maximal" filtered albumin 1.85/84 is used, it would appear that reabsorption of filtered albumin occurs in the control periods, but during dextran diuresis again more albumin is recovered in the urine than can be accounted for by this hypothetical filtration calculation.

In contrast to columns A and B, column C indicates the result obtained if one assumes that the albumin recovered in the urine represents at least the minimal amount filtered (27) (assuming no tubular secretion of albumin). In this case, the quantity of albumin filtered per 100 ml. of filtered water appears to *increase* during dextran diuresis. This result again does not necessarily imply change in glomerular permeability, since it is based on the relatively greater increase in excreted albumin over filtered water. It does not take account of either change in serum albumin concentration or tubular reabsorption of albumin.

The formula C/B relates albumin excreted per unit of water filtered to a fraction of the serum albumin concentration which is assumed to be filtrable normally. The use of such a ratio only serves to emphasize that any calculation of glomerular permeability must at least take account of these simultaneous variables. If C/B is an expression of glomerular permeability to albumin, it is apparent from the values derived that the relative permeability to albumin may be increased 3 to 5 times during dextran diuresis independently of that for water.

Other phenomena relating to plasma proteins also may be explored. For example, the existence of a pool of mobile plasma protein in tissue about as large as that in the plasma was suggested some time ago (28). An extravascular "pool" of plasma proteins in nuclei and cytoplasm of many organs and in the interstices of connective tissue has recently been demonstrated (29). These extravascular sources presumably were drawn upon to replace the albumin lost in the urine during dextran induced diuresis, and thereby limited the reduction of circulating albumin. However, with the continuous proteinuria observed during the nephrotic syndrome, both concentration and total circulating albumin are reduced. Apparently this homeostatic mechanism is more responsive to acute changes in plasma protein content. Since the extravascular sources of plasma protein appear to be available in children who have had the nephrotic syndrome for some time, reduction in concentration or quantity of circulating albumin does not signify exhaustion of these stores.

The increased excretion of water with dextran infusions appears to initiate increased excretion of solutes, essentially sodium and chloride. This is not always observed, as demonstrated by Greenman and associates (30). Their patients, however, were maintained on severe dietary sodium restriction (2 mEq. per day); our patients were ingesting 30 to 50 mEq. of sodium per day. Since osmolarity of the already expanded extracellular water was either unchanged or decreased, and hypertonic solutes were not infused, the hypothesis of cellular dehydration (31) seems an unlikely explanation for the increased excretion of sodium and chloride. Since the quantity of sodium filtered per minute appeared to be either decreased or unchanged, increased sodium excretion obviously resulted from decreased tubular reabsorption of this ion. A similar phenomenon was observed by Strauss, Davis, Rosenbaum, and Rossmeisl during hypotonic expansion of extracellular fluid volume in recumbent subjects (32). They postulated that the locus of action of the expanded volume is in the cephalad portion of the body.

The actual mechanism by which the renal tubular reabsorption of salt is modified, however, remains unexplained.

The precise mechanism of the observed water diuresis also remains undetermined. Unresponsiveness of renal tubules to antidiuretic hormone during dextran diuresis seems excluded since intravenous injection of pitressin produced antidiuresis. The total solute concentration of the extracellular fluid was usually reduced during dextran infusion and diuresis. In two instances, this was not observed although profound diureses occurred. In five other patients, the acute reduction of total solute concentration in the extracellular water approximated 4 to 6 per cent. Verney (33) and Baldes and Smirk (34) showed that a rapid diminution in osmolarity of the extracellular water of 1.5 to 2 per cent initiates diuresis, presumably through inhibited elaboration of antidiuretic substances (A.D.S.) mediated *via* osmoreceptors. Other hypothetical possibilities also could account for the observed dextran-induced water diuresis.

SUMMARY

1. The intravenous infusion of hyperoncotic (12 per cent) dextran into children with the nephrotic syndrome often initiates a water diuresis followed by increased urinary excretion of sodium and chloride with removal of most of the edema fluid. Transient hypertension is the most common complication of dextran infusion. The safety of repeated infusions is not yet ascertained.

2. Diuresis was associated with a marked increase of plasma volume and with increased effective renal plasma flow in seven patients in whom it was measured; however, glomerular filtration rate was increased in only two of the four instances in which it was simultaneously measured.

3. At its maximum, the water diuresis was characterized by a urinary total solute concentration of less than 100 mOsm. per L., and a thiosulfate U/P ratio approximating 10. This

maximal diuresis could be transiently inhibited by intravenous injection of pitressin. The exact cause of the water diuresis initiated by dextran infusion has not been determined.

4. Observations during dextran infusion in four children suggested that the calculated permeability of the glomerular membranes to albumin (a) is relatively increased in the nephrotic syndrome as previously demonstrated by others, (b) may increase further during dextran infusion, and (c) appears to be independent of that for water. These interpretations depend upon the method of calculation applied to the observed excretion of albumin.

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FAT INTAKE, SERUM CHOLESTEROL CONCENTRATION, AND ATHEROSCLEROSIS IN THE SOUTH AFRICAN BANTU. PART I. LOW FAT INTAKE AND THE AGE TREND OF SERUM CHOLESTEROL CONCENTRATION IN THE SOUTH AFRICAN BANTU[1]

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During the past few decades, interest in the effects of overnutrition and undernutrition on blood cholesterol values, and their bearing on the pathogenesis of atherosclerosis, has led to the carrying out of numerous experimental studies on both animals and man (1, 2). Several studies have shown that diets characterized by reduced fat intake, are associated with, or give rise to reductions in serum cholesterol levels (3, 4). Dealing specifically with changes in fat intake, Keys, Mickelsen, Miller, and Chapman (5), using iso-caloric diets, found that when fat intake is substantially altered, well-marked changes appear in serum cholesterol concentration, which, moreover, are independent of cholesterol intake.

Confirmation of the correlation between fat intake and cholesterol values is afforded by two types of evidence. Firstly, populations subjected to dietary restrictions, which invariably include reduced fat intake, have been shown to have lower cholesterol levels than when the relevant conditions returned to normal. Thus, in the last war, very low cholesterol values were observed among the inmates of various European concentration camps (6–8). Conversely, a significant rise in mean blood cholesterol level occurred in groups of German subjects from 1947 to 1949, this change being attributed to dietary improvements, including increased fat intake (9).

The second type of evidence is provided by populations habituated to a high fat intake, *e.g.*, the inhabitants of the United States (10) and Denmark (11), who have higher mean serum cholesterol values than peoples accustomed to a lower fat intake, *e.g.*, population groups in Italy (12), Spain (13), China (14), India (15), Belgian Congo (16), Southern Rhodesia (17), and Bechuanaland (18).

In addition to the observation that serum cholesterol level varies directly with fat intake, the evidence indicates that the extent of the rise in serum cholesterol which occurs with age is influenced by the amount of fat customarily consumed. Thus, Keys and his co-workers (10) examined a series of American adult males habituated to a high fat intake (that of the nation as a whole supplies a mean of 38 per cent of calories), and observed a sharp regular rise in cholesterol levels from youth to late middle age (51 to 60 years), followed by a recession of values. On the other hand, with other workers, Keys (13) investigated the serum cholesterol age trend of a comparable series of Italians, who are used, *inter alia*, to a much lower fat intake (the mean national intake supplies about 20 per cent of calories); they found that the mean serum cholesterol level began on approximately the same plane, rose until the middle thirties when a plateau was reached (12).

This last investigation stimulated us to determine the age trend of serum cholesterol concentration in groups of the South African Bantu, whose average intake of fat is less than a half of that usual in the United States, and also less than that consumed by the Italian groups cited.

MATERIAL

Fat Intake

Among the Bantu of Southern Africa, maize, wheat, and, in certain areas, "kaffir corn" (*sorghum*), together with small amounts of legumes such as "cow peas" (*Vigna unguiculata*), "sugar beans" (*Phaseolus vulgaris*), "Jugo beans" (*Voandzeia subterrannea*) are the staple sources of calories: the fat intake is low (19–23). In Johannesburg in 1941, Fox and Janisch (20) carried out a study of African income and expenditure in 987 families,

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and reported an average daily consumption of 35 g. fat, which supplied 17 per cent of the mean estimated calorie intake. In a study carried out in 1951, du Toit (23) investigated the food intake of 100 families in an urban Bantu township in Springs, near Johannesburg. Families were selected to constitute a random sample, and observations extended over three months. The mean fat intake per man unit *per diem* was 45 g., which supplied 14 per cent of mean estimated calorie intake. (Daily diet provided a mean of 2,950 calories, and 84 g. gross protein.) There is additional information on the food habits of these urban and peri-urban Bantu population groups, which, although based on less factual data, confirms the low proportion of calories, certainly less than 20 per cent, which are provided by dietary fat.

Subjects

Bantu subjects used for determining the age trend of serum cholesterol. We found it impossible to collect blood from persons pursuing their every-day life in the Bantu townships around Johannesburg, the chief reason being the reluctance of non-hospitalized natives to give blood. Our subjects were chosen from patients attending Baragwanath Non-European Hospital (1,350 beds), which serves Johannesburg and the surrounding townships. The 218 persons examined included 167 men and 51 women, whose ages ranged from 15 to 93 years. Subjects were mainly ambulant out-patients awaiting treatment for minor surgical lesions, *e.g.*, hydrocele, wounds, otorhinolaryngal complaints, skin diseases, etc.; in the light of present knowledge these conditions are not considered likely to seriously affect general nutritional state or serum cholesterol values. The number of elderly persons attending out-patients departments, unfortunately, is small; the majority of such subjects were patients with cataract just admitted to hospital for operative treatment. All subjects were in fair state of nutrition; there were no cases of undernourishment or emaciation.

Bantu subjects from other South African territories (*a*) *Bechuanaland.* The diet of the Tswana of South Bechuanaland resembles that consumed by urban Bantu. Reports by Squires (24, 25), together with our own impressions, suggest that the consumption of "kaffir corn" is probably higher, and possibly that larger amounts of meat and vegetables are consumed. The average amount of fat eaten is considered to be of the same order as in urban areas. The above reports indicate that, speaking generally, the state of nutrition is reasonably good. At Lobatsi and Kenya, blood samples were obtained from 38 subjects, *i.e.*, 23 male and 15 female subjects, their ages ranging from 21 to 38 years, mean age being 30 years. Of the male subjects, 10 were prison laborers, and 10 were agricultural workers; the remaining three males and all the females were attending out-patients departments (the female subjects mainly on account of their children) in the two centers mentioned. All subjects were in outward good health.

(*b*) *Basutoland Bantu.* The indigenous Basuto consume a diet of similar pattern to that of the urban Bantu, except that their consumption of maize is higher and of wheat lower compared with town dwellers (26). The intake of fat is not believed to be different. In Johannesburg, the Witwatersrand Native Labour Association Headquarters is the clearing station through which pass annually about a quarter of a million Bantu mineworkers before being directed to the different mines, where they work for recurrent periods of about nine months. Blood samples were obtained from 31 subjects immediately after their arrival from Basutoland. Ages ranged from 21 to 40 years, mean age being 29 years. All subjects were passed as medically fit.

Bantu consuming a partially Europeanized diet with higher fat content. Serum cholesterol values were determined on 69 Bantu adult males from two local centers of employment which provided rations for their workers. The diets, although still containing a large amount of cereal products, differed from those usually consumed mainly on account of their higher energy and higher fat contents, the latter being about 65 to 80 g. *per diem* and providing 20 to 25 per cent of estimated calorie intake. Ages ranged from 21 to 40 years, mean age being 31 years. Subjects had been used to the diet for a minimum of one month, though usually for a longer period. All subjects were medically fit.

European control subjects. The European subjects examined were either on the staff of this Institute, or were blood donors to the South African Blood Transfusion Service. All persons were in a good state of health. Two age groups were selected for study. The first, 21 to 30 years, numbered 55 subjects, with mean age 28 years. The second group 41 to 50 years, with mean age 46 years, was composed of 46 subjects. Unfortunately, no recent dietary surveys have been undertaken on local Europeans. In the family Budget Inquiry (27) carried out in 1936, the middle income groups dwelling in Johannesburg consumed a mean daily fat intake of 108 g. which supplied roughly 36 per cent of the estimated calorie intake. In le Riche's study in Pretoria (28), the lower class European group consumed a mean of 91 g. fat *per diem*, which provided about 24 per cent of estimated calorie intake. Our impressions are that the fat consumption of our European subjects differed little from that of the corresponding strata of population found in the United States, *i.e.*, fat intake supplied at least 30 to 35 per cent of calorie intake.

Time of Sampling

Blood was collected approximately mid-way between breakfast and lunch time, except that about half of the bloods of the Bantu consuming the Europeanized diet were collected between noon and 4 p.m.

METHODS OF ANALYSIS

Serum cholesterol. Serum was shaken with the usual Bloor mixture, and after evaporation, the extracted cholesterol was determined by the Liebermann-Burchard reaction, the final color being stabilized as described by

TABLE I

Serum cholesterol data on Johannesburg Bantu of different age groups *

Age groups (*years*)	Average age (*years*)	No. of subjects	Range of serum cholesterol	Mean and S.D. values	Minnesota data	
					Average age	Mean and S.D. values
20	18	25	99–224	155±32	18	168±31
21–30	26	45	88–236	166±28	25	184±34
31–40	38	40	92–265	169±40	35	200±43
41–50	45	54	85–240	179±37†	45	236±37†
51–60	54	28	73–252	184±39†	55	256±46†
60+	68	26	103–283	183±42†	70	225±42†

* Cholesterol values given in mg. cholesterol per 100 ml. serum.
† Means differ significantly ($P < 0.01$).

Sheftel (29). To obviate any uncertainty, the method employed will be described in detail.

All glassware is thoroughly dried beforehand. Into a 15 ml. stoppered centrifuge tube are put 8 ml. absolute alcohol and 3 ml. ether; 0.2 ml. serum is added slowly. After shaking for one min., the tube is allowed to lie horizontally for one hour, and then centrifuged for 10 min. at 3,000 r.p.m. The supernatant liquor is poured into a small flat-bottomed flask, and the alcohol-ether mixture evaporated off at low temperature on a sand bath. When cool, 5 ml. dry chloroform are added, the precipitate dissolved, and the solution poured into a small glass stoppered reagent bottle. The standard solution, 5 ml. chloroform containing 0.4 mg. cholesterol (prepared from stock chloroform solution containing 100 mg. per cent cholesterol), is poured into a similar bottle. To each bottle is added 1 ml. glacial acetic acid and then 2 ml. acetic anhydride containing 10 per cent sulphuric acid (this mixture is freshly prepared on each occasion and cooled before use). The green colors produced are allowed to develop in the dark for 30 min., and measured in a Hilger Spekker Spectrophotometer using Spectrum Red filter 608.

The method followed records all color as cholesterol. Hence, all values are very slightly too high because the color intensity of cholesterol esters is greater than that of free cholesterol (29).

Using this method, average difference between 100 duplicates was 5.8 mg. cholesterol, per 100 ml. serum. Fifty determinations were carried out in parallel with Dr. W. M. Politzer of the Routine Biochemistry Department of this Institute, using the same method. The average difference between results was 6.2 mg. cholesterol per 100 ml. serum. Finally, on taking five sera of cholesterol value of about 150 to 170 mg. per cent, and adding pure cholesterol sufficient to double this value, the mean recovery rate was 98 per cent (range 95 to 101 per cent). With five sera having values about 300 to 320 mg. per cent, and treated correspondingly, mean recovery of added cholesterol was 97 per cent(range 94 to 102 per cent).

Liver function tests. Determinations were made of thymol turbidity (30), cephalin-cholesterol flocculation (31), Takata Ara (32), and gamma globulin (33).

TABLE II

Serum cholesterol data on different Bantu and South African European groups

Race	Age group (*years*)	Average age (*years*)	No. of subjects	Cholesterol values
Bechuanas	21–38	30	38	149±36* (86–210)
Basutos	21–40	29	31	153±37* (94–221)
Johannesburg Bantu group	21–40	31	85	167±33* (88–265)
Europeanized Bantu group	21–40	31	69	178±36* (100–249)
South African Europeans	21–30	28	55	206±35 (145–322)
	41–50	46	46	238±38 (186–328)

* The means of Bechuanas and Basutos are significantly lower than that of the Johannesburg Europeanized Bantu ($P < 0.05$).

RESULTS

The results are summarized in Tables I to III.

COMMENT [2]

The American data cited refer to values obtained when subjects were at basal condition. According to the findings of Keys and his co-workers (10) the values we obtained are likely to be approximately 4 mg. per cent higher than at basal state.

The age trend of serum cholesterol concentration in the Bantu. Below 40 years, there is no statistical difference between the mean cholesterol values of the Bantu and American age groups. After 40

[2] Statistical analysis of our results was undertaken by Mr. Walter Lutz B.A. of the Department of Mathematics of the Witwatersrand University.

TABLE III

Liver function data on different Bantu groups

	Bechuanas	Basutos	Europeanized urban Bantu group*
No. of subjects	38	31	57
Per cent showing liver dysfunction	68	71	65
Mean serum cholesterol in dysfunction group	151±37	151±39	180±37
Mean serum cholesterol in group not showing dysfunction	145±34	155±36	175±35

* The liver function tests on the Johannesburg Bantu consuming the Europeanized diet were undertaken by Dr. I. Bersohn and Miss C. D. Sussman, B.Sc. of the Diagnostic Department of this Institute.

The liver function data were obtained in an endeavor to learn whether there was any correlation between presence of liver dysfunction and level of cholesterol concentration. Initially, a subject was judged to show liver dysfunction when the thymol turbidity value exceeded 2 units, the cephalin cholesterol (24 hour reading) and Takata Ara tests were positive, and the gamma globulin concentration exceeded 1.25 g. per 100 ml. serum. Since this classification revealed dysfunction present in 80 to 90 per cent of subjects, a re-grading was carried out in which the thymol turbidity ceiling level was raised to 3 units, and the gamma globulin to 1.40 g. per 100 ml. serum. Subjects with liver function data equal to or below the stated values were considered to show no or mild liver dysfunction; the remainder, for the purposes of argument, were regarded as having marked dysfunction.

years, the means of the Bantu groups are significantly lower than the American means ($P < 0.01$).

The mean annual increments were computed as the slopes of the least squares regression lines fitted to the data over age groups showing no significant curvilinearity, *i.e.,* 17 to 45 years. The slopes differ significantly ($P < 0.01$). The rises in serum cholesterol per year are 0.378 ± 0.89, and 2.29 ± 0.180 mg. per 100 ml. serum, respectively, for the Bantu and Minnesota groups. There is no significant difference between the corresponding origins, 141.7 and 127.7 mg. per cent respectively.

Cholesterol values of other Bantu groups. There is no significant difference between mean cholesterol values of the 21 to 40 age group of the Johannesburg Bantu, the Bechuanas, and the Basutos.

The mean cholesterol value of the 21 to 40 year group of the urban Bantu consuming the Europeanized diet is significantly higher than the values for the Bechuanas and Basutos ($P < 0.05$), although not for the Johannesburg Bantu.

Cholesterol values of the European groups. The serum cholesterol value of the 21 to 30 year group of South African Europeans, although slightly higher, does not differ significantly from the corresponding American value. Likewise, our 41 to 50 year group does not differ from the American group significantly in mean serum cholesterol value.

Liver function tests. Among the three Bantu groups examined (Table III) there is no significant difference between the mean cholesterol values of the sections showing little or no dysfunction, and those showing marked dysfunction.

DISCUSSION

It will be apparent that the low fat intake of the South African Bantu correlates with their low serum cholesterol values. The factors that may share in the responsibility for the correlation will now be considered.

Race

Populations, both White and Non-White, who are habituated to a low fat diet are marked by low serum cholesterol levels. Furthermore, Snapper (14) noted low values in poor Chinese in Peiping, yet high values among commercial Chinese dwelling in Indonesia. In addition, Verhoef (34) reported low values among indigenous Javanese, compared with normal values occurring in Javanese who had been resident in Holland for some time. Our Europeanized Bantu group on their higher fat intake, had a higher mean cholesterol concentration in the serum compared with the two rural Bantu groups consuming their usual low fat diet. Bearing these points in mind, we agree with van Oye and Charles (16) in doubting whether the racial factor has any relevance in the present issue.

State of health

Subjects in the main Bantu group examined either were attending or were recently admitted to hospital. Hence, understandably, their cholesterol data may be treated with some reserve. Nevertheless, our results reveal no significant difference between the serum cholesterol concentrations of the 21 to 40 year age groups of the hospital Bantu on the one hand, and the Bechuanas, and Basutos on the other. Since all three groups were believed to be accustomed to much the same pattern of diet, and since the two rural groups were

known to be in a good state of outward health, it would seem that the complaints for which the Johannesburg Bantu were attending hospital were not markedly affecting their serum cholesterol figures. It is therefore probable that the age trend of serum cholesterol values observed in the hospital Bantu group is likely to be equally valid for Non-hospitalized Bantu pursuing their usual avocations.

It is apparent that every-day good health as experienced by the Bechuana and Basuto subjects is compatible with low serum cholesterol concentrations.

Dietary factors

Fat intake. At first sight, it would seem reasonable to consider that the low fat intake of these people is likely to be primarily responsible for the low cholesterol values observed. There are, however, a number of observations which preclude the unreserved acceptance of this view: (*a*) The serum cholesterol value of the Johannesburg Bantu on the higher fat diet was less affected by the increase in fat intake than was anticipated: actually, there was no significant difference between the mean cholesterol value and that of the Johannesburg Bantu consuming their usual low fat diet; (*b*) In a comprehensive Dutch study (35), it was found that a diet of almost exclusively vegetable products had the effect of reducing the mean cholesterol value of 20 subjects, mean age 38 ± 8 years, from 260 to 200 mg. per cent in three months, although the diet was rich in vegetable fat—a mean of 108 g. *per diem,* providing 36 per cent of calories; (*c*) In a further recent Dutch study (36), the mean serum cholesterol value of 55 strict vegetarians ("vegans"), of mean age 39 years, was found to be 166 mg. per cent, although, here again, the fat content of the diet provided no less than 34 per cent of calorie consumption. In studies (*b*) and (*c*), it is clear that a factor or factors other than reduced or low fat intake were in operation. While there is little doubt that the low fat intake of the Bantu is implicated in the low cholesterol values recorded, present knowledge allows no preciseness of incrimination.

Cholesterol intake. Certainly the cholesterol content of the habitual diet of the Bantu is low in comparison to the levels of intake that are common overseas. In America, the comprehensive studies of Keys, Mickelsen, Miller, and Chapman (5, 13) have demonstrated that alterations in cholesterol intake have virtually no influence on serum cholesterol value. Whether such results would be equally demonstrated in the Bantu pattern of diet, we are not in a position to say.

Caloric deficiency. Keys and his associates (4) have critically examined reported data on blood lipids in acute and semi-starvation. They consider that the latter produces little if any change in blood lipid levels except for a decrease in cholesterol, which contrasts with the suggestive slight increase in this component in acute starvation. Unfortunately, there is little information available on cholesterol levels accompanying mild degrees of caloric insufficiency such as occur occasionally at certain seasons with the Bantu of Southern Africa. As noted earlier, upon altering fat intake *only* in diets of iso-caloric content, Keys, Mickelsen, Miller, and Chapman (5) obtained highly significant changes in serum cholesterol concentration. But the influence of alterations in caloric intake *only* in diets of iso-fat content has been less thoroughly investigated. Walker (37) has recently studied changes in various blood lipid components of subjects during weight reduction on diets already low in fat but of reduced calorie content; he observed significant changes in certain lipo-protein fractions, although not in cholesterol level. On this account we question whether the possibly lower plane of caloric nutrition exhibited by the subjects in this series is of relevance in regard to the low cholesterol values observed.

Pattern of diet. In relevant publications, of the dietary factors considered, almost exclusive attention has been devoted to the rôles of fat, cholesterol, and calorie intakes, in influencing serum cholesterol concentrations. The *pattern of diet,* however, habitual to population groups displaying hypocholesterolemia, is distinctive, and has not been given the attention that it deserves. During war-time, it is frequent for diets to fall in energy value, protein (particularly animal protein), and fat (particularly cholesterol) contents, and to rise in carbohydrate and crude fiber contents. The consumption of meat, dairy produce, eggs, fatty foods and sugar usually goes down, but increases occur in consumption levels of cereals and their products, legumes, and vegetables. Speaking

generally, this pattern of diet is much the same as that of Non-White dwellers in tropical and semi-tropical regions. One feature of the diet described which may be of relevance is that it usually contains a large amount of crude fibre, thereby constituting a "high residue" diet, which contrasts with a "low residue" diet, *i.e.*, one based on refined foodstuffs. In this respect, attention is drawn to the two Dutch investigations cited above (35, 36); both diets consumed, which were relatively high in fat, although associated with reduced or low mean cholesterol values, were high in bulk-forming capacity. We must point out that knowledge of the effect of pattern of diet on cholesterol synthesis, excretion into the bile, absorption and re-absorption in the intestine, and loss in the stools, is virtually nil. Investigations on certain of these aspects have been initiated.

Summarizing, of the dietary factors, our impression is that, apart from low fat intake, there is a factor or factors in the pattern of diet of the Bantu possibly related to its high fibre content which bears some responsibility for the low serum cholesterol values observed.

Pathology and dysfunction of the liver

Hypocholesterolemia is the rule in advanced forms of cirrhosis (38), a lesion commonly observed among the South African Bantu, as with many other indigenous populations of tropical and semi-tropical countries (39–41). Local observers have the impression that cirrhosis is present in approximately a tenth of the adult hospitalized urban Bantu population. Moreover, intermediate degrees of chronic liver disease are common (42), and certain liver function tests have revealed widespread abnormalities affecting more than half of the Bantu population (43). The question naturally arises, to what extent this commonness of hepatic disease and dysfunction is responsible for the low serum cholesterol values observed. Unfortunately, present evidence does not permit a satisfactory answer to be given. However, regarding cirrhosis, we doubt whether its incidence is sufficiently high to account for our results. In relation to milder hepatic damage and dysfunction, no information is available upon the extent to which such states affect serum cholesterol values, although a detailed investigation on this subject is now in progress at this Institute (43). Two of our findings, however, appear to be relevant. Firstly, among the 69 Bechuanas and Basutos, the biochemical tests undertaken revealed abnormalities in 48 (67 per cent); yet there was no significant difference in mean cholesterol levels between the groups with and without dysfunction. Furthermore, of the Bantu group consuming the Europeanized diet, 37 of the 57 subjects examined (65 per cent) showed abnormality of liver function according to the criteria used; yet the groups with and without dysfunction had mean cholesterol concentrations which did not differ significantly (Table III). We therefore suggest that hepatic dysfunction and mild chronic liver disease are little implicated in the low serum cholesterol values obtained.

Pancreatic disease

Marked atrophy of the parenchymal cells of the pancreas is common among Bantu infants and young children, but such lesions are found to be milder and much less frequent in adults (44). As far as we are aware, no correlation has been postulated between the presence of such lesions and altered serum cholesterol concentrations. Hypercholesterolemia is a feature of diabetes, but the latter occurs very infrequently among the Bantu population generally (39, 41). We thus consider it unlikely that pancreatic disease is implicated in the low serum cholesterol data obtained.

Non-dietary factors

Both Keys (13) and Groen, Tjiong, Kamminga, and Willebrands (35) have referred to subjective factors, such as mental over-exertion, emotional tension, *etc.*, in promoting hypercholesterolemia. We do not know to what extent these factors in the Bantu influence their cholesterol levels.

The uncertainties involved in the subject under discussion are clearly apparent and indicate the extent of the further investigational work required. Nevertheless, we feel justified in believing that an important influencing factor sharing responsibility for the low serum cholesterol concentrations observed in the Bantu is their habitually low fat intake. In addition, however, we have the impression that there is a factor in the pattern of diet,

possibly its bulk-forming capacity, which bears some responsibility.

SUMMARY

1. The South African Bantu, in common with most indigenous populations dwelling in tropical and semi-tropical regions, are habituated to a diet, *inter alia*, of low fat content, averaging less than a half of that consumed in the United States.

2. The age trend of serum cholesterol has been investigated in 218 urban Bantu subjects, of ages ranging from 15 to 93 years. Up to 40 years, the differences between mean values obtained among the Bantu and in Minnesota (Keys and his associates), are not significant; thereafter, the Bantu data are significantly lower ($P < 0.01$). The regression curves of the Bantu and Minnesota data for the group from 17 to 45 years differ significantly in slope ($P < 0.05$).

3. Groups of rural Bantu from other territories likewise accustomed to a low fat intake, and in a good state of health, were also found to have low mean cholesterol values.

4. Urban Bantu consuming a Europeanized diet with an increased fat intake have a significantly higher mean serum cholesterol concentration than corresponding groups of rural (though not urban) Bantu eating their usual diet with its low fat content ($P < 0.05$).

5. Discussion of the relevant evidence suggests that racial differences, state of health, caloric deficiency, low cholesterol intake, and pathology and dysfunction of liver and pancreas bear little responsibility for the low serum cholesterol values observed.

6. The main dietary influencing factor may well be the habitually low fat intake of these people. Impressions suggest, however, that further dietary factor or factors are implicated, possibly related to the high "residue" diet of these people.

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FAT INTAKE, SERUM CHOLESTEROL CONCENTRATION, AND ATHEROSCLEROSIS IN THE SOUTH AFRICAN BANTU. PART II. ATHEROSCLEROSIS AND CORONARY ARTERY DISEASE

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No vital statistics are available for the Bantu of South Africa, and knowledge of their disease pattern is dependent on clinical impressions and on examination of hospital material, which is, of necessity, selected. The numerous fallacies involved in the interpretation of hospital statistics, especially for heart disease, have recently been reviewed by Mainland (1). Unfortunately, however, such sources at present provide the only adequate information available for the Bantu.

There is, however, widespread clinical belief that the more severe complications of atherosclerosis, *i.e.*, coronary and cerebral thrombosis, angina pectoris and intermittent claudication, are rare among Africans. This impression may be due partly to the small number of Africans living to an advanced age. For example, in 1951 only 17 per cent of the patients (excluding children) attending Baragwanath Non-European Hospital were found to be over 45 years of age, in contrast to the figure of 47 per cent for Europeans at the Johannesburg General Hospital. A sample of the population from the native township of Alexandra near Johannesburg in 1953, has shown that rather less than 8 per cent are over 50 years of age (2). That the lower longevity of Africans is not the only reason, however, is suggested by the work of other authors. In Rhodesia, Gelfand (3, 4) has reported that, in his experience, coronary artery disease is very infrequent. Severe atheroma and coronary artery disease have been recorded as uncommon in Uganda (5). In a comprehensive review of post-mortem material among the Bantu in Johannesburg, Becker (6) concluded that coronary thrombosis with infarction was rare.

MATERIAL

The subjects in the present study were in-patients dying in Baragwanath Hospital, the largest general hospital serving mainly the Bantu in Johannesburg and surrounding townships of the Witwatersrand. In the local study of Alexandra Native township (2) from which many of these patients are drawn, it was found that practically all the Bantu population were prepared to attend a Western hospital when seriously ill. There is no evidence of bias in the attendance of cardiac patients in the general hospitals in the Johannesburg area.

Post-mortem examinations are obtained in approximately one-third of the general patients dying at this hospital. The majority of patients on whom a necropsy is not performed are surgical cases, infants and young children. Apart from these cases, the major reason for not doing a post-mortem, is failure to obtain permission from a relative. We have no evidence that this permission is biased for any particular disease or section of the Bantu community. Cases where there is factual evidence of an unnatural cause of death such as motor accidents and stabbings, are sent to the Government Medico-Legal Laboratories. In 1953, approximately one-third of the deaths registered in the Johannesburg magisterial district were from this hospital, but the exact figure is not known as some patients may go from hospital to their birthplace in the country, if seriously ill, and death certificates are not corrected to district of domicile.

In relation to atherosclerosis, there may be some bias due to the following cause. All clinicians at this hospital agree that if coronary artery disease is suspected in a Bantu out-patient, every attempt is made to secure his admission due to the suspected rarity of the condition. This factor accordingly tends to increase rather than decrease the proportion of patients with severe coronary atherosclerosis seen at post-mortem.

METHODS

Coronary artery and aortic atherosclerosis. A series of 523 unselected necropsies, performed under the supervision of one pathologist at Baragwanath Hospital, were available for study, with adequate records on the condition of the aorta and coronary vessels. The primary cause of death for these patients is given in Table I. It should also be pointed out, however, that many showed, in addition, histological lesions in the liver of the type described in a previous paper from this hospital (7), although not showing clinical evidence of hepatic disease. For comparative purposes an attempt was made to follow the classification of atherosclerosis described by Wanscher, Clemmesen, and Nielsen (8) in Denmark.

Grade I. Cases with no obvious naked-eye lesions, and those with only slight fatty streaking and minimal atherosclerosis in the aorta and/or coronary arteries.

Grade II. A more marked degree of atherosclerosis with the plaques tending to be confluent, but without obvious ulceration, or significant diminution of the lumen of the coronary arteries.

Grade III. Severe atherosclerosis of aorta, including ulceration and calcification, but in which atherosclerosis of the coronary arteries does not significantly diminish the lumen.

Grade IV. Cases with marked atherosclerosis of the coronary arteries.

These cases are frequently but not invariably associated with well-marked aortic disease.

As in the Danish series, cases of aneurysm and syphilitic aortitis have been excluded. Further, cases of malignant disease have been omitted as in the Danish series, since the latter authors have demonstrated a markedly reduced incidence of severe atherosclerosis in cancer patients in contrast to patients dying without cancer.

The above classification essentially depends on the general appearance on naked-eye examination of the arteries, especially the aorta. The main disadvantages are the high degree of subjectivity, and the failure to allow for differences in the degree of atherosclerosis between the coronary arteries and the aorta. In our experience the degree of atherosclerosis in each is not necessarily comparable. Accordingly, while the classification in Grades I to III corresponds reasonably closely to that described by the Danish authors, being chiefly dependent on the condition of the aorta, this is not true for Grade IV, in which we have included all cases with severe coronary disease, even if the degree of aortic change was slight.

In our series the coronary arteries were examined by opening them along their major branches, so that direct comparison with the excellent studies of White, Edwards, Dry, and Ackerman (9, 10) in Minnesota was not possible. It can be stated, however, by correlation of naked-eye and microscopic appearances, that if the coronary arteries showed plaques approximately of Grade II atherosclerosis or less according to the classification illustrated by these authors (9), they were not graded as Grade IV above in our classification. Only those cases, in which more than one of the atheromatous plaques present appeared of greater severity, were placed in Grade IV in this series. Although this grading was done by naked-eye examination, in nearly all Grade III and Grade IV cases a section from the coronary arteries was available.

The Danish authors described the coronary arteries in Grade IV as being "transformed to stiff and narrow chalky pipes without elasticity." It is our opinion that not a single case graded as IV by us, could be justifiably so described. Accordingly, we have attempted to be as critical

TABLE I

Cause of death as established at necropsy

Primary cause of death	Men	Women	Total
Cardio-vascular disease (including tuberculous pericarditis)	84	66	150
Tuberculosis	42	23	65
Central nervous system disease	47	23	70
Respiratory disease	39	18	57
Miscellaneous bacterial and parasitic diseases	18	21	39
Genito-urinary disease	26	38	64
Primary liver disease	12	6	18
Diabetes mellitus	3	4	7
Gastro-intestinal disease	14	4	18
Miscellaneous	15	20	35
Total	300	223	523

TABLE II

Incidence of atherosclerosis at necropsy

Age group (years)	Grade I No.	I %	II No.	II %	III No.	III %	IV No.	IV %	Total
a. In 300 adult men									
20–29	46	85	6	11	1	2	1	2	54
30–39	57	77	16	22	1	1	0		74
40–49	50	65	24	31	2	3	1	1	77
50–59	14	32.5	26	60.5	2	5	1	2	43
60–69	15	37	19	46	3	7	4	10	41
70+	3	27	7	64	0		1	9	11
									300
b. In 223 adult women									
20–29	59	98	1	2	—		—		60
30–39	41	79	11	21	—		—		52
40–49	25	61	16	39	—		—		41
50–59	8	30	15	55	3	11	1	4	27
60–69	6	24	14	56	5	20	—		25
70+	2	11	11	61	4	22	1	6	18
									223

TABLE III

Comparison: Grade III and IV atherosclerosis in Bantu and Danish series

Age group	Grade III Bantu %	Grade III Danish %	Grade III P. %	Grade IV Bantu %	Grade IV Danish %	Grade IV P. %
a. Men						
40–49	3	32	<1	1	8	Not significant
50–59	5	50	<1	2	10	Not significant
60–69	7	45	<1	10	21	<1
70+	0	48	<1	9	37	<1
b. Women						
40–49	0	20	Not significant	0	0	Not significant
50–59	11	32	Not significant	4	6	Not significant
60–69	20	46	<1	0	21	<1
70+	22	42	<1	6	45.5	<1

TABLE IV

Distribution of cases dying from coronary diseases in relation to total necropsies and cardiac deaths

Age group	Total post-mortems	Cardiac deaths	Coronary disease	Per cent coronary deaths out of total %
		a. Men		
20–29	124	27	0	—
30–39	193	50	0	—
40–49	212	62	1	0.5
50–59	121	34	1	0.8
60–69	103	21	0	—
70+	42	18	2	5
Unknown	12	2	0	—
	807	214	4	0.5
		b. Women		
20–29	129	17	0	—
30–39	122	34	0	—
40–49	89	27	0	—
50–59	73	16	1	1.5
60–69	52	19	0	—
70+	45	14	3	4.5
Unknown	11	1	0	—
	521	128	4	0.6

as possible of our data, and it is probable that several cases graded as III or IV in this series would have been graded less by the Danish workers.

Table II gives the number of cases in each grade. In Table III the number of cases graded as III and IV are compared with the corresponding Danish groups.

The degree of nutrition is probably lower than in the Minnesota series (10), in that only 1.7 per cent of the males and 10.7 per cent of the females were described in our protocols as stout or obese, whereas 39.4 per cent of males and 33.3 per cent of females were considered poorly nourished. In the Minnesota series 38.8 per cent of the females were described as over-weight and 22.3 per cent under weight.

Coronary artery disease. In a larger series of 1,328 consecutive necropsies carried out by various members of the staff at the same hospital over a period of five and one-half years and which includes the previous series, there were seven cases of coronary thrombosis or myocardial infarction, and one case in which the diagnosis of ischemic heart disease was considered tenable.

In Table IV is shown the distribution of these cases in relation to the total number of post-mortem and cardiac deaths.

COMMENTS

In common with other African reports (5, 6), the number of cases in the older age groups is small in both our series.

As already mentioned a classification as used in Tables II and III is unsatisfactory due to the high degree of subjectivity. But, as the criteria for severe atheroma (Grade III and IV) are clearly stated by the Danish authors (8), we believe that, in the absence of a more satisfactory classification, a comparison between these two groups is justified with the following qualification. Of this series of 523 post-mortems, in no aorta was a degree of atherosclerosis found comparable to that described as Grade IV aortic disease by the Danish authors, and our cases were placed in this category on account of the condition of the coronary arteries. To exclude the possibility that this observation was not due to differences of interpretation, we compared samples of our aortas in the older age groups with a group of unselected aortas from South African European subjects in the same age groups, kindly put at our disposal by Dr. B. J. P. Becker of this Institute. These European subjects were from the Johannesburg General Hospital. The crude death rate from arteriosclerotic and degenerative heart disease for the white population of South Africa was 153.6 per 100,000 of the population in 1949, in contrast to 268.1 in the United States (11). The severe degrees of atherosclerosis observed in the majority of these European aortas have not been seen by us in Bantu subjects.

The value of statistical analysis for such data, nevertheless, is doubtful and has only been calculated at the 1 per cent level using the method described by Hald (12). However, the difference between the Danish and Bantu figures (Table III) is sufficiently marked to indicate that severe atherosclerosis is relatively infrequent among the Bantu. These data which depend mainly on the aorta, are supported by our findings in the coronary arteries, although the relationship between atherosclerosis of the coronary arteries and aorta is not absolute. Gordon, Bland, and White (13), in a series of 3,400 post-mortems from Massachussetts General Hospital, found that 12.8 per cent of the cases in the 41 to 60 age group and 13.3 per cent in the 61 to 80 age group, showed occlusion of the coronary arteries. The corresponding figures in our series were 1.6 per cent in the 40 to 59 age group, and 3 per cent in the over 60 age group.

In the Minnesota series (9) previously mentioned, over 60 per cent of hearts from male cases over 50 years of age showed sclerosis with 50 per

cent closure of the lumen in some part of the coronary system. In our study we considered that only 7 per cent of male hearts in this age group showed atherosclerotic plaques of this severity. This cannot be stated with absolute certainty, however, due to the different method of examination, but we do not consider it probable that we have missed coronary narrowing of this degree.

That these observations may be valid is supported by the low frequency of deaths from coronary artery disease observed at this hospital. From Minneapolis, in a series of 22,149 post-mortems from non-diabetic patients, Clawson and Bell (14) found that coronary artery disease was the major cause of death in 10.7 per cent of male and in 5.3 per cent of female deaths between 50 and 69 years of age. The corresponding figures at Baragwanath Hospital are 0.45 per cent and 0.8 per cent respectively (Table IV).

In Minnesota, Clawson (15) ascribed 24 per cent of cases of death from non-congenital heart disease, including all age groups, primarily to coronary disease. This proportion, of course, is influenced by the age composition of the hospital population. In our total series (Table IV), however, only 5.7 per cent of cardiac deaths in patients over 50 years of age were primarily due to coronary disease.

Our results are in conformity with those of Becker (6) who found only one Bantu death due to coronary thrombosis among a series of 352 predominantly Bantu post-mortems over the age of 50 years. Although not conclusive, these observations suggest that severe atherosclerosis of the aorta and coronary arteries are less frequent in the hospitalized Bantu in the Witwatersrand of South Africa than among the American and Danish hospital populations.

DISCUSSION

Assuming our conclusion to be valid, namely that the incidence of severe atherosclerosis in the Bantu is lower than in Europe and America, there are two possible explanations. In these people, atherosclerosis has developed more slowly, or secondly, the atherosclerotic lesions have regressed before death.

We are not aware of any pathological confirmation of regression taking place in severe atherosclerosis in man. However, regression may be invoked as an explanation of the lower incidence of severe atherosclerosis in cancer patients than in non-cancer patients observed by Wanscher, Clemmesen, and Nielsen (8), also for the lower incidence reported in under weight patients from the United States (10, 16).

In regard to the genesis of atherosclerosis, it is now generally accepted that many factors are implicated, and recent work has reemphasized the importance of local vascular lesions such as intimal thrombosis and fibrosis, also medial degeneration (17–20). No detailed study of local vascular lesions has as yet been reported in the Bantu, but one of us (W. J. P.) in a series of 150 consecutive necropsies has observed some degree of medial degeneration in the aorta in every case over 20 years of age (21). Further, fibro-elastic thickening and elastic degeneration of the intima as described by Moon and Rinehart (20) have been observed in all adult patients in which the coronary arteries were examined microscopically. However, the quantitative relationship of these factors to the development of atherosclerosis in the Bantu has not been elucidated.

The influence of race must also be considered in view of the observations of Blache and Handler (22) who, in discussing the generally accepted lower incidence of coronary artery disease in the American Negro in contrast to the white American, have also shown that local degenerative changes in the coronary vessels develop more slowly in the negro. It is difficult, however, to explain the differences in the number of deaths from atherosclerosis in closely related races or even changes in mortality in the same race at different periods as described by Morrison (23), if local lesions are to be postulated as the sole cause of atherosclerosis. We would accordingly agree with Gutman who considers lipid metabolism of basic importance in the development of atherosclerosis (24), and prefer at present to regard the low frequency of severe atherosclerosis in the Bantu as possibly due to metabolic factors, rather than entirely due to racial and local vascular changes.

As stated in Part I of this study (25), fat intake and serum cholesterol concentration and possibly habituation to a refined low residue diet are regarded as factors influencing the development of severe atherosclerosis. In Europe and America it has not been possible to produce direct evidence

in man on the exact rôle of fat intake and serum cholesterol levels in the pathogenesis and severity of atherosclerosis. In primitive communities the acquisition of statistics from a migrating and poorly controlled population is much more difficult than in Western countries, and, for the reasons stated, it has not been possible in this investigation to utilize comparable hospital groups. We believe, however, that our studies have demonstrated that Bantu hospital patients drawn from a population habituated to a low fat high residue diet have a lower incidence of severe atherosclerosis than Danish and American hospital populations; secondly, a series of out-patients at the same hospital also have lower serum cholesterol concentrations than groups taken from the general population in Denmark and the United States. Further work is being continued at this Institute on the comparative development of atherosclerosis in the Bantu and European races in South Africa, with special regard to the influence of local vascular lesions, and general differences in diet and metabolism.

SUMMARY

An examination of post-mortem material at Baragwanath Non-European Hospital shows that severe atherosclerosis and coronary artery disease are less common among Bantu patients attending this hospital, than in American and Danish hospital populations.

Some of the possible aetiological factors causing this lower incidence of atherosclerosis have been briefly mentioned. Attention is drawn to the low level of serum cholesterol among out-patients and in-patients attending this hospital reported in the first part of this study (25). These observations are in accord with the belief that populations with a low serum cholesterol tend to have a lower incidence of severe atherosclerosis.

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THE OXYGEN DISSOCIATION CURVE IN ANEMIA OF VARIOUS TYPES [1]

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It is a clinical observation of long standing that the patient who has become anemic gradually, is often well compensated for the lower hemoglobin level he possesses. Thus it is not uncommon for an individual with a hemoglobin level well below 50 per cent, due to pernicious anemia or hypochromic anemia, to carry on his normal activities. It is generally believed that the compensatory adjustment is in part due to increased utilization of oxygen by the tissues and in part to circulatory adaption. It has also been suggested, from studies of the gas transport of red cells in anemia (1–4), that it may in part be due to displacement of the oxygen dissociation curve to the right of normal whereby the amount of oxygen released to the tissues is increased; the number of patients observed in these investigations is small probably because of the difficulties and time-consuming nature of the methods. The fullest report is that of Richards and Strauss (2) who indicated a shift to the right of the oxygen dissociation curve apparent, however, only at the abnormal plasma pH of 7.64. Only the reports of Dill and his coworkers (3) and Isac, Matthes, and Yamanaka (4), demonstrate a shift to the right of the oxygen dissociation curve at the standard plasma pH of 7.4. There are no observations, as far as we are aware, on the oxygen dissociation curve at various stages after recovery from anemia. Many of the patients in these early reports had received whole blood transfusions before the blood gas studies and, as we have shown, this produces an alteration in the oxygen dissociation curve of the anemic recipient (5). Various hypotheses, many of them contradictory, have been offered regarding the cause of the beneficial shift of the oxygen dissociation curve in anemia but none of them affords a satisfactory explanation for the phenomenon.

For these reasons a further and more extensive study of the oxygen dissociation curve in anemia appeared warranted. The present paper describes the oxygen dissociation curves of twenty-nine individuals suffering from various types of anemia. Observations on the position of the oxygen dissociation curves at various times during and after recovery from the anemia are also presented.

MATERIAL AND METHODS

The subjects of this study comprised eleven patients with megaloblastic anemia in relapse and three in therapeutic remission, three patients with nutritional hypochromic anemia, three patients with hypochromic anemia secondary to chronic hemorrhage, two patients with aplastic anemia, four patients with anemia associated with chronic nephritis, acute leukemia, reticulosis, and scurvy respectively, and three patients with hemolytic anemia. Many of the observations were made before specific therapy was administered and subsequent curves were performed at different periods during and after recovery. None of the patients had received blood transfusions before the blood gas studies were performed, with the exception of one patient with aplastic anemia of long standing (Table II, Case 12).

The collection of blood samples, the equilibration in tonometers with the desired gas tensions, the gas analyses, and the plasma pH (pHs) determinations were performed on venous blood samples removed without stasis by the methods described elsewhere (5). The subjects were resting for at least one hour before the removal of the blood samples. The cell pH (pHc) was determined from the Henderson-Hasselbalch equation given below. In the case of the severely anemic subjects the blood samples were first concentrated by removal of plasma under oil to bring the packed cell volume approximately to normal.

$$pHc = pK'c + \log \frac{(BHCO_3)\ blood}{(H_2CO_3)\ blood}$$

where $(BHCO_3)$ blood = the total CO_2 of oxygenated blood at 40 mm. Hg partial pressure of CO_2 and (H_2CO_3) blood = H_2CO_3 content of blood calculated from the formula $(H_2CO_3) = a\ (pCO_2)$ where "a" is the solubility coefficient of carbon dioxide in blood (from data

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[2] Enabled to undertake this work by scholarships from the E.C.A. for Greece and from the University of Thessaloniki.

TABLE I

Normal subjects and megaloblastic anemia [1]

Case no.	Patient	Date	R.B.C. mill./cu. mm.	HbO_2 Vol. %	Color index	Packed cell volume	$\frac{HbO_2}{P.C.V.}$	Retics. %	CO Vol. %	$(CO_2)_a$ Vol. %	$(CO_2)_o$ Vol. %	$\Delta(CO_2)_{a-o}$	Blood pH_a	Blood pH_o	pO_2 for Hb = HbO_2			Comments
															Blood pH_a and pH_o	$pH_a = 7.4$	$pH_o = 7.22$	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	
	6 normal subjects			18–21 19.5					0.25–0.7 0.5	50–62.5 57.5	42–52 48	8–11 9.5	7.33–7.43 7.39	7.17–7.26 7.23	25–28 26.5	25.5–27 26.5	25.5–27 26.5	Mean normal values from 6 individuals with range figures
1	J. McQ. Male 79 yrs.	26/9/53 After concentration	2.2	9.5 16	1.1	23 38	0.41	1	0	65	57 49	8 16	7.47	7.22	28 28	30.5	28	Addisonian pernicious anemia. Parenteral vitamin B_{12} therapy started on 27/9/53
2	M. P. Female 30 yrs.	2/4/53 After concentration	0.90	5.5 19	1.5	10.5 38	0.52	1	0	57	50 41	7 16	7.4	7.14	32 32	32	28.5	Megaloblastic anemia with free HCl in gastric juice. Curve before treatment
3	M. M. Female 70 yrs.	9/5/53 After concentration	2.05	12 18	1.4	24 39	0.50	3	0.2	59	53 49	6 9.5	7.42	7.23	27 27	27.5	27	Addisonian pernicious anemia. Parenteral liver extract therapy started on 11/5/53
4	J. B. Male 56 yrs.	13/1/53 26/1/53 29/1/53 18/2/53	1.39 2.79 3.14 3.60	7.5 12 13 16.5	1.3 1.1 1.1 1.1	22 29 38	0.34 0.41 0.44	14 2.5 3.0 1	0.2	59 57	 47	 10	7.42 7.40	 7.20	31 30 31 28.5	31.5 28.5	 28	Addisonian pernicious anemia. Parenteral vitamin B_{12} therapy started on 6/1/53
5	J. A. Male 36 yrs.	23/2/53 28/2/53 17/3/53	1.51 1.85 3.38	8 10 14.5	1.3 1.4 1.1	18 25 40	0.44 0.40 0.36	2.5 31.5 2.5	0.5 1	51 62	47 50	4 12	7.35 7.44	 7.24	28 26.5 26.5	26.5 28	 28.5	Addisonian pernicious anemia. Parenteral vitamin B_{12} started on 23/2/53
6	A. D. Female 43 yrs.	8/2/53 17/2/53 6/3/53 16/3/53 17/3/53	2.3 2.62 3.06 3.42 3.51	10 12 15.5 14.5 14.5	1.1 1.1 1.1 1.1 1.0	19.5 30	0.51 0.48	20 3.6 1	0.2 0.25	52 56 62	48 48.5 52	4 7.5 10	7.36 7.39 7.44	 	29 29 29 31 30	27.5 31 31.5	 	Addisonian pernicious anemia. Parenteral vitamin B_{12} started on 2/2/53

[1] Column D—HbO_2 vol. % = the oxyhemoglobin capacity in volume per cent.
Column J—$(CO_2)_a$ vol. % = the total carbon dioxide of true plasma from oxygenated blood at a CO_2 partial pressure of 40 mm. Hg.
Column K—$(CO_2)_o$ vol. % = the total carbon dioxide of oxygenated blood at a CO_2 partial pressure of 40 mm. Hg.
Column L—$\Delta(CO_2)_{a-o}$ = the difference between the total CO_2 of true plasma and oxygenated blood.
Column M—Blood pH_a = the plasma pH determined on true plasma from oxygenated blood at a CO_2 partial pressure of 40 mm. Hg.
Column N—Blood pH_o = the cell pH determined on oxygenated blood at a CO_2 partial pressure of 40 mm. Hg.
Columns O, P, Q—pO_2 for Hb = HbO_2 = the partial pressure of oxygen at which oxyhemoglobin saturation is 50% at the plasma pH and cell pH of the subject (column O), at standard plasma pH (column P), and at standard cell pH (column Q).

TABLE I—*Continued*

Case no.	Patient	Date	R.B.C. *mill./cu. mm.*	HbO_2 *Vol. %*	Color index	Packed cell volume	$\frac{HbO_2}{P.C.V.}$	Retics. %	CO *Vol. %*	$(CO_2)_s$ *Vol. %*	$(CO_2)_o$ *Vol. %*	$\Delta(CO_2)_{s-o}$	Blood pH_s	Blood pH_o	pO_2 for Hb = HbO_2			Comments
															Blood pH_s and pH_o	$pH_s = 7.4$	$pH_o = 7.22$	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	
7	M. C. Female 58 yrs.	3/3/53 16/3/53	1.77 1.47	8.5 8.5	1.2 1.5	19 18	0.44 0.47	2	0	62 58.5	56 53	6 4.5	7.44 7.41		30 29	31.5 29.5		Addisonian pernicious anemia. Oral therapy with Hog's stomach and vitamin B_{12} started on 9/3/53
8	J. L. Male 67 yrs.	1/9/52 8/9/52 7/11/52 5/2/53	1.75 2.6 4.49 5.02	11.5 14.5 20 20	1.6 1.4 1.1 1.0	23 31 43 46	0.50 0.47 0.47 0.44	5.2 12	0.25 0.5	66 57	58.5 45	7.5 12	7.47 7.40		30 30 27.5 27	32 27		Addisonian pernicious anemia. Oral therapy with Hog's stomach and vitamin B_{12} started on 2/9/53. Subsequent parenteral vitamin B_{12}
9	M. F. Female 51 yrs.	23/7/52 24/7/52 1/8/52 28/8/52 24/2/53	1.43 1.5 3.83 3.82	7.8 7.5 9.5 15.7 18	1.4 1.6 1.0 1.2	16 16 21 33 41.5	0.49 0.47 0.45 0.44 0.45	2.2 1 20.4 1	0.2	59 58	54 53	5 5	7.41 7.40		28.5 28.5 28.5 28.5 27	29 28.5		Addisonian pernicious anemia. Oral therapy with Hog's stomach and vitamin B_{12} started on 25/7/52 and continued during period of study
10	M. R. Female 59 yrs.	8/8/52 14/8/52 5/9/52	2.22 2.32 3.8	10 10.2 17	1.1 1.1 1.1	21.5 24 36.5	0.47 0.43 0.46	1 18.5 1	0	62 64	56 58	6 6	7.44 7.45		28.5 28.5 29	30 30.5		Addisonian pernicious anemia. Parenteral vitamin B_{12} started on 9/8/53
11	A. N. Female 43 yrs.	19/1/53 30/1/53 17/2/53	2.91 3.69 4.21	13.3 17.3 17.3	1.2 1.2 1.0	38 39	0.45 0.44	14.8 2.8 1	0 0.5	59	54	5	7.42		31 31 28.5	31.5		Addisonian pernicious anemia with subacute combined degeneration of cord. Parenteral vitamin B_{12} started on 9/1/53
12	M. D. Female 40 yrs.	6/3/53	4.11	18	1.1	39	0.46	1	0.5	57	48	9	7.40	7.22	27	27	27	Addisonian pernicious anemia. Maintenance therapy with parenteral vitamin B_{12} for 3 months
13	A. B. Male 78 yrs.	27/1/53	4.83	20.5	1.0	47	0.44		0.5	55.5	45	10.5	7.38	7.20	27	26.5	26.5	Addisonian pernicious anemia. Maintenance therapy with parenteral liver extract and vitamin B_{12} since August, 1948
14	J. E. B. Male 85 yrs.	28/1/53	4.55	20	1.0	43	0.46		0.5						26			Addisonian pernicious anemia. Maintenance therapy with parenteral vitamin B_{12} since May, 1951

TABLE II

Hypochromic anemia, secondary anemia, aplastic anemia and hemolytic anemia

Case no.	Patient	Date	R.B.C. *mill./cu. mm.*	HbO_2 *Vol. %*	Color index	Packed cell volume	$\frac{HbO_2}{P.C.V.}$	Retics. %	CO *Vol. %*	$(CO_2)_a$ *Vol. %*	$(CO_2)_o$ *Vol. %*	$(CO_2)_{a-o}$	Blood pH_a	Blood pH_o	pO_2 for Hb = HbO_2			Comments
															Blood pH_a and pH_o	pH_a = 7.4	pH_o = 7.22	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	
1	J. McL. Male 68 yrs.	27/2/53 After concentration	3.77	8 15	0.53	21 39	0.38	2	0.5	57.5	51 42	6.5 15.5	7.4	7.16	29 29	29	27	Nutritional hypochromic anemia
2	M. B. Female 41 yrs.	13/2/53	2.71	7	0.65	18	0.40	2	0	54	47	7	7.37		30	29		Nutritional hypochromic anemia
3	J. D. Male 70 yrs.	16/3/53	2.92	10	0.85	23	0.43	2	0.2	58	50	8	7.41		32	32		Nutritional hypochromic anemia
4	A. W. Female 51 yrs.	23/2/53 After concentration	3.1	9 15	0.73	25 37.5	0.36	2	0.2	52	47 42	5 10	7.36	7.12	32 31.5	30	28	Hypochromic anemia secondary to hereditary telangiectasis
		18/3/53	3.58	13.5		33				58	50	8	7.4		34	34		
		24/3/53	3.79	15.2						55	47	8	7.38		32	31		
5	W. McC. Male 33 yrs.	24/2/53	3.10	8.5	0.70	22	0.40	2	0.2	52	47	5	7.35		27	25		Hypochromic anemia secondary to hemorrhoids
6	W. M. Male 42 yrs.	15/9/53	3.42	7	0.51	19	0.36	1.3	0	59	54	5	7.41		32	32.5		Hypochromic anemia secondary to hemorrhoids
7	A. F. Male 73 yrs.	5/3/53	2.15	9.5	1.11	24	0.40	12	0	56	52	4	7.39		28.5	28.5		Severe scurvy due to dietary inadequacy
8	J. S. Male 28 yrs.	19/2/53	1.85	7	0.95	13	0.54	5	0	34	31	3	7.14		32	25		Chronic glomerulonephritis. Blood urea 240 mg. %

TABLE II—*Continued*

Case no.	Patient	Date	R.B.C. *mill./cu. mm.*	HbO_2 *Vol. %*	Color index	Packed cell volume	$\frac{HbO_2}{P.C.V.}$	Retics. %	CO *Vol. %*	$(CO_2)_a$ *Vol. %*	$(CO_2)_o$ *Vol. %*	$(CO_2)_{a-o}$	Blood pH_a	Blood pH_o	pO_2 for Hb = HbO_2			Comments
															Blood pH_a and pH_o	pH_a = 7.4	pH_o = 7.22	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	
9	C. R. Male 73 yrs.	24/4/53 After concentration	2.17	9 16	1.03	20 36	0.45	5	0.2	56	48.5 44	7.5 12	7.39	7.15	32 32	31.5	28.5	Reticulosis
10	D. A. Male 16 yrs.	7/4/53	1.51	7.5	1.25	14	0.53	1	0	63	57	6	7.44		29.5	31		Acute leukemia
11	J. McG. Male 16 yrs.	4/3/53 After concentration	1.76	9 18.5	1.27	20 40	0.45	1	0.2	53	48.5 43	4.5 10	7.37	7.17	28 28	27	26	Aplastic anemia of recent onset
12	M. T. Female 53 yrs.	25/5/53 After concentration	1.82	7.5 17	1.04	16 36	0.47	1	0.2	59.5	55 47	4.5 12.5	7.42	7.17	30 30	31	28	Aplastic anemia of several years' duration. Numerous transfusions
13	C. M. Female 56 yrs.	6/7/53 After concentration	2.24	10 17.5	1.0	22 39	0.45	12.5	0.2	59	52 45	7 14	7.41	7.16	28	28.5 28.5	26	Congenital acholuric jaundice. Microspherocytosis prominent
14	I. N. Male 8 yrs.	10/4/53	3.86	17.5	1.14	39	0.45	10	0	75	47.5	27.5	7.4	7.21	27	27	26.5	Congenital acholuric jaundice. Microspherocytosis prominent
15	A. W. Female 29 yrs.	8/6/53	1.06	5.5	1.34	11	0.50	20	0.25	52	50	2	7.36		27	25.5		Acquired hemolytic anemia. Microspherocytosis prominent

TABLE III

Oxyhemoglobin saturation of laked blood solutions derived from patients with anemia

	Normal blood	Pernicious anemia in relapse (Table I, Case 8)	Hypochromic anemia (Table II, Case 4)	Pernicious anemia after treatment (Table I, Case 10)
pO_2 *mm. Hg*		HbO_2 *Volume %*		
15	28	29	29	28
20	50	50	48	50.5
30	78	80	78	79
40	86	87	85	85

by Dill in the paper of Keys, Hall, and Barron (6) and Dill, Graybiel, Hurtado, and Taquini (7)).

pK'c was obtained from the alignment chart and procedure given in the paper of Keys, Hall, and Barron (6) modified in such a manner for the pK'c to be 5.98 for normal blood (8, 9) giving a cell pH of 7.20 to 7.25. The other values for pK'c derived from this alignment chart were changed proportionately.

Correction of the position of the curve according to the plasma pH and cell pH was carried out in curves drawn on logarithmic co-ordinates by a slight modification (10) of the procedure outlined by Dill in the paper of Keys, Hall, and Barron (6).

Buffered solutions of laked blood of pH 7.4 were prepared according to the methods described by Brooks (11) and Darling and Roughton (12).

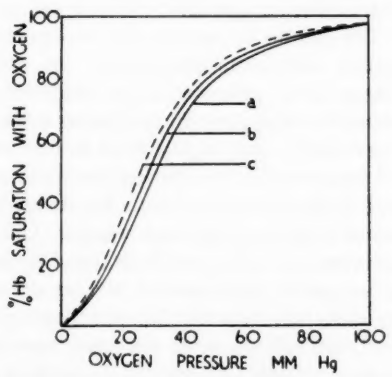

FIG. 1. OXYGEN DISSOCIATION CURVES FROM ANEMIC AND NORMAL SUBJECTS

Curve a—Addisonian pernicious anemia in relapse, hypochromic anemia, secondary anemia. At serum pH 7.4

Curve b—Addisonian pernicious anemia in relapse, hypochromic anemia, secondary anemia. At cell pH 7.22

Curve c—Normal blood at serum pH 7.4 and cell pH 7.22

RESULTS

The results are presented in Tables I, II, and III and in Figure 1. The position of the oxygen dissociation curve is indicated by the partial pressure of oxygen at which 50 per cent of the hemoglobin is oxygenated.

In Table I are shown the mean values obtained from six normal individuals, which have been presented in detail elsewhere (10), and the individual data of eleven patients with megaloblastic anemia in relapse and three in therapeutic remission. Of the patients in relapse the position of the oxygen dissociation curve at the plasma and cell pH of the subject (column O) was displaced to the right of normal in all but one (case 3). After correction to the standard plasma pH of 7.4 (Column P and curve a of Figure 1) this shift to the right was more pronounced except in cases 5 and 6. In these two cases the curve was nearly normal at standard plasma pH. Serial observations (cases 4, 8-11) show that the displacement to the right of the oxygen dissociation curve did not alter during the earlier response to treatment, although by the time the red cell count had reached about the four million per cubic mm. level, between four and six weeks, some lessening of the displacement was apparent. In the two patients in whom the initial oxygen dissociation curves were normal at standard plasma pH (cases 5 and 6) a shift to the right developed during the early stages of treatment. The presence of reticulocytes (column H) did not appear to influence the position of the oxygen dissociation curve. The curve was normal in position in the three patients in therapeutic remission (cases 12–14). The cell pH (column N) was determined in seven of the cases, of whom three were in relapse, two were during treatment, and two were in therapeutic remission. Of the cases in relapse, the difference between the cell and plasma pH was increased in those showing a shift of the oxygen dissociation curve to the right at standard plasma pH (cases 1 and 2), and normal in the case showing a normal oxygen dissociation curve at standard plasma pH (case 3). In the patients (cases 4 and 5) in whom the cell pH was determined when the red cell count had reached between three and four millions per cubic mm., at which time the oxygen dissociation curve was still slightly displaced to

the right at standard plasma pH, the difference between the cell and plasma pH was normal. After correction to standard cell pH the position of the oxygen dissociation curve was unaltered (column Q). The cell pH was normal in the two patients in therapeutic remission (cases 12 and 13) both of whom showed a normal position of the oxygen dissociation curve.

In Table II are shown the results obtained in patients with nutritional hypochromic anemia, chronic post-hemorrhagic anemia, secondary anemia, aplastic anemia, and hemolytic anemia. With the exception of the case of anemia associated with chronic nephritis (case 8), in which there was an acidosis, the plasma pH of the subjects was normal; in the majority of cases the values were in the lower range of normal. The position of the oxygen dissociation curve at the plasma and cell pH of the subject was displaced to the right of normal in all the patients with the exception of one patient with post-hemorrhagic anemia (case 5), one patient with aplastic anemia (case 11), and all three patients with hemolytic anemia (cases 13–15). The displacement to the right was still present after correction to the standard plasma pH of 7.4 (Figure 1, curve a) excepting the patient with chronic nephritis whose curve was to the right of normal at blood plasma pH but normal in position at standard plasma pH. The cell pH was low in six of the seven cases in which it was determined and normal in the remaining case (case 14), a patient with hemolytic anemia. After correction to the standard cell pH the position of the oxygen dissociation curve was normal in the two patients with hemolytic anemia (cases 13, 14) and in one patient with aplastic anemia (case 11) but was still slightly to the right (Figure 1, curve b) in the patients with hypochromic anemia (cases 1, 4), secondary anemia (case 9), and the remaining patient with aplastic anemia (case 12).

Table III shows that the oxygen dissociation curves of hemoglobin solutions (buffered solutions of laked blood) from patients with pernicious anemia and hypochromic anemia are the same as that of hemoglobin solutions of normal blood.

DISCUSSION

Odaira (1) states that in anemia there is a marked acidosis and that as a result there is a shift to the right of the oxygen dissociation curve. The techniques employed by Odaira are open to criticism, however, and many authors (3, 4, 13–16) have failed to demonstrate an acidosis in pernicious anemia but have, in fact, shown a tendency to an alkalosis. Stadie and Martin (17), obtained a normal oxygen dissociation curve in a single patient with pernicious anemia but they do not give the plasma pH at which the curve was performed. Richards and Strauss (2) reported that the oxygen dissociation curves in six patients with pernicious anemia and five patients with secondary anemia were in the normal position at the normal plasma pH of the blood (7.4), and that only at the abnormal plasma pH of 7.64 was there a shift to the right. Several of the cases studied by Richards and Strauss had received transfusions of whole blood within a few days prior to the determination of the oxygen dissociation curves. In discussing the cause of the shift observed at plasma pH 7.64, they suggest that it could be due, in the patients with pernicious anemia, to an increased concentration of hemoglobin within the red cell, which would have the result, at this high plasma pH, of increasing the value $(-\log r)$ as developed by Van Slyke, Wu, and McLean (18, 19) and so increasing the difference between the plasma and cell pH. The hypothesis is admitted to be invalid in hypochromic anemia in which there is a shift of the oxygen dissociation curve to the right. Dill and his co-workers (3) report that in one patient with pernicious anemia the oxygen dissociation curve performed before treatment was normal but the subsequent curves during treatment with liver orally, and single curves in four other cases of pernicious anemia during treatment, were displaced to the right of normal. No curves were determined after recovery from anemia. Dill and his co-workers by indirect methods found the value rH to be smaller than normal and so conclude that the difference between plasma and cell pH is increased, the cell pH being relatively more acid than normal. Both Dill and his co-workers and Henderson (20) were unable to relate the observed shift in the oxygen dissociation curve to altered osmotic or electrolytic relationships between cell and plasma. Isac, Matthes, and Yamanaka (4) found that the oxygen dissociation curves in four patients with pernicious anemia, four patients with secondary anemia, one patient with aplastic anemia, and one patient with hemolytic

anemia were displaced to the right of normal. These authors, using indirect methods like Dill and his co-workers, were unable to demonstrate any decrease in the cell pH.

The present observations confirm that there is a shift to the right of the oxygen dissociation curve at standard plasma pH in the majority of patients with hypochromic anemia, secondary anemia, and megaloblastic anemia in relapse, thus providing for the anemic patient an increased yield of oxygen to the tissues. The time taken for the shift in the curve to lessen following treatment was in the patients with megaloblastic anemia some four to six weeks but it should be noted that many of these patients were the subjects of an investigation of the value of oral vitamin B_{12} in the therapy of pernicious anemia and were not always showing optimal hematological responses. It is not apparent why two of the patients with megaloblastic anemia had a normal oxygen dissociation curve initially but developed a shift to the right during the course of treatment. A similar development was observed by Dill and his co-workers. The present investigation does not confirm that there is a shift to the right of the oxygen dissociation curve in hemolytic anemia (4).

The beneficial effect of the displacement of the oxygen dissociation curve is obvious but the mechanism responsible for it is not apparent. A reduction of the plasma pH of the subject would displace the curve to the right but there is no evidence that acidosis is present and moreover the displacement of the curve is still present after correction to standard plasma pH. The presence of carboxyhemoglobin causes a shift to the left of the oxygen dissociation curve (17) and the position of the oxygen dissociation curve in the normal subject may be influenced to a small extent by the carboxyhemoglobin present (21). We have found smaller amounts of carboxyhemoglobin in the anemic subjects than in normal subjects but the quantities involved are so small that they could not produce the observed displacement of the oxygen dissociation curve. Alteration in the concentration of hemoglobin within the red cell also cannot be the factor responsible for the displacement of the oxygen dissociation curve since the displacement is demonstrable in both pernicious anemia and hemoglobin deficiency anemia. The effect of the increased plasma volume in anemia can likewise be excluded as the cause of the displacement since artificial dilution with fresh plasma from the same individual does not influence the position of the oxygen dissociation curve (2, 5).

It is now accepted (22, 23) that many different kinds of hemoglobin, at least some of which may influence the position of the oxygen dissociation curve, may be present in pathological states. It is unlikely, however, that an abnormality of hemoglobin can be responsible for the observed displacement of the oxygen dissociation curve in anemia since the oxygen dissociation curves of hemoglobin solutions of anemic blood are the same as those of normal blood.

The position of the oxygen dissociation curve is dependent basically upon the cell pH and it is unfortunate that there is no entirely satisfactory method by which the cell pH can be determined directly. Hampson and Maizels (24) using a direct method at room temperature (glass electrode determinations on red cells hemolysed by repeated exposure to low temperature) found that the difference between the cell pH and plasma pH in pernicious anemia was greater than normal while in hypochromic anemia it was less than normal. Maizels (25), in referring to cell pH determinations, expresses doubts about his results partly because of the many manipulations involved and partly because the determinations were made at room temperature in conditions far removed from those in which red cells exist in the body. The indirect method for determining cell pH, in which the value "r" as developed by Van Slyke, Wu, and McLean (18) ($-\log r = pH_s - PH_c$) is determined, or the nomogram developed by Dill (see 6) is used, is superior to the direct method particularly as regards comparative studies, although the Henderson-Hasselbalch equation is not a strict physical chemical equation. If the indirect method, however, is applied to anemic blood where the cell phase is small, there is the disadvantage that experimental errors are multiplied. To avoid this disadvantage the cell pH was determined after concentrating the anemic blood samples by removal of plasma under oil to bring the packed cell volume into the normal range. This manipulation permits more accurate study.

Using this indirect method the cell pH was low relative to the plasma pH in cases of pernicious anemia in relapse and cases of hypochromic anemia

which showed a displacement of the oxygen dissociation curve to the right. On the other hand, in cases of pernicious anemia in therapeutic remission having the oxygen dissociation curve normal in position, the cell pH was normal. The cell pH was also normal in the case of pernicious anemia in relapse which showed a normal oxygen dissociation curve. In the cases in which the cell pH was low correction of the oxygen dissociation curve to the standard cell pH largely, but not completely, corrected the displacement to the right, suggesting that while a low cell pH is the main factor in producing the displacement there is also a further factor. In both cases of aplastic anemia the cell pH was also found to be low. Correction of the position of the oxygen dissociation curve in these cases to the standard cell pH wholly eliminated the displacement of the curve in the case of recent onset and partly corrected it in the case of longstanding. This patient had received numerous blood transfusions over several years and it would be unwise to draw any conclusions from examining this individual's oxygen dissociation curve since it would appear that one was in fact examining the oxygen dissociation curve of many donors. The cell pH was low in one of the two cases of hemolytic anemia in which the cell pH was determined and normal in the other. In the former case, correction of the position of the curve to the standard cell pH wholly corrected the slight displacement of the oxygen dissociation curve that was present at the cell pH of the subject. The cases of pernicious anemia studied during recovery showed that the oxygen dissociation curve is still slightly abnormal when the red cell count is about the 4 million per cubic mm. level, at which time there is still a degree of macrocytosis. In the cases in complete therapeutic remission, where there is no macrocytosis, there is no displacement of the oxygen dissociation curve.

While a lowered cell pH thus accounts for the major part of the displacement of the oxygen dissociation curve the results suggest that there is in addition a further factor. Measurements of the red cell diameter thickness ratio were not made in this study but the observed relationship between displacement of the oxygen dissociation curve and shape of the red cell in stored red blood cells (5, 10) indicates that such measurements might usefully be done in further studies in gas transport in anemia.

SUMMARY

1. The oxygen dissociation curves in twenty-nine cases of anemia have been studied.

2. In ten of the eleven cases of megaloblastic anemia in relapse and in nine of the ten cases of hypochromic anemia and secondary anemia there was a slight displacement of the oxygen dissociation curve to the right. No shift to the right was present in three cases of hemolytic anemia or in one of two cases of aplastic anemia.

3. The displacement of the oxygen dissociation curve persisted during the period of recovery in the cases of megaloblastic anemia but was not present in cases in therapeutic remission. The position of the curve was not influenced by the presence of reticulocytes.

4. The major part of the displacement appears to be due to an alteration in the cell pH.

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SIMULTANEOUS DETERMINATIONS OF THE TOTAL VOLUME OF RED BLOOD CELLS BY USE OF CARBON MONOXIDE AND CHROMIUM^{51} IN HEALTHY AND DISEASED HUMAN SUBJECTS [1]

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During recent years several methods have been introduced for estimating the total volume of circulating erythrocytes by measuring dilution in the blood stream of cells which have been tagged with radioactive phosphorus, iron, or chromium (1–6). Values for red cell volume thus obtained may be considered more reliable than indirect estimates based on hematocrit and "plasma volume" as measured with T-1824 or other substances which label plasma proteins, because no error is introduced by the discrepancy between venous (or arterial) and "body" hematocrits (7–9). For the same reason, a closer approximation to the true total blood volume is found by adding together red cell and "plasma" volumes, as measured independently, than is found by use of the plasma volume-hematocrit methods (10).

Erythrocytes may also be labeled with carbon monoxide gas, and this is the basis of methods which have been employed widely to estimate blood volume and total circulating hemoglobin (11–17). It has long been suspected that the volume of distribution of carbon monoxide in the body (henceforth to be called "CO-available volume") may be larger than true blood volume, since the gas has affinity for pigments which are located extravascularly, such as myoglobin or non-circulating hemoglobin (11, 18, 19). Previous work has shown a discrepancy between CO-available volume and blood volume as measured with radioactively tagged cells, both in man (9) and in the dog (18). The difference in magnitude between the reported results in man and dog, and the inadequacies of the methods used in the human experiments, leave the problem open to further exploration. For these reasons, and because the CO method has certain practical advantages for physiologic and clinical work, it seemed desirable to make comparative measurements of the apparent volume of erythrocytes in healthy and diseased human subjects, using simultaneously a CO method which has been employed extensively in this laboratory and a method utilizing Cr^{51}.

MATERIALS AND METHODS

The subjects were six healthy volunteers and 29 hospitalized patients with various diseases (Table I). Determinations of CO-available volume and of the dilution of radioactively tagged cells were carried out simultaneously as a rule, but occasionally in sequence, during the same forenoon. All subjects had fasted overnight and had been recumbent for a minimum of 30 minutes before procedures were begun.

The method used for estimating CO-available volume has been described fully (14, 15). Carbon monoxide was delivered into a small, closed system into which the subject rebreathed for 20 minutes, O_2 being added as required. Samples of blood taken before and at the end of this period were analyzed for CO by the palladium reduction method (20). No correction was made for the residual CO in the rebreathing system, since this has been shown to account for an error of not more than 1 per cent of the final result (14).

The method described by Sterling and Gray for determination of Cr^{51} space (6) was modified as follows: On the afternoon prior to the study, 15 ml. of the subject's blood was drawn into a syringe containing heparin and injected into a sterile, stoppered centrifuge tube. To this was added sufficient sodium chromate solution containing Cr^{51} [3] to give a total of 25,000 to 30,000 counts per second as measured in a scintillation counter.

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[2] While carrying out this work, Dr. Nomof was a Trainee in Cardiology of the National Heart Institute, U. S. Public Health Service.

[3] The Cr^{51} was made at Oak Ridge and processed by Abbott Laboratories as 0.1 per cent sodium chromate solu-

TABLE I

Comparison of apparent RBC volumes obtained by CO and CR^{51} methods used simultaneously

1 Subject		2 Age	3 Sex	4 Clinical state	5 Weight (*Kg.*)	6 Height (*cm.*)	7 Hematocrit 20 min. CO sample	8 Hematocrit 20 min. Cr^{51} sample	9 Vol. RBC by CO (*liters*)	10 Vol. RBC by Cr^{51} (*liters*)	11 Ratio CO/Cr^{51}*
				Group I—No Disease							
1	D. P.	29	M	Healthy	62.0	164	49.1	46.7	2.03	1.58	1.28
2	D. G.	26	M	Healthy	68.2	171	42.4	42.2	2.07	1.69	1.23
3	S. G.	21	M	Healthy	53.8	173	46.0	46.5	2.07	1.93	1.07
4	P. S.	22	M	Healthy	73.4	178	38.4	38.7	2.65	2.03	1.30
5	J. M.	23	M	Healthy	80.6	175	42.6	41.6	2.64	2.08	1.27
6	J. H.	43	M	Healthy	77.3	174	46.2	46.6	2.66	2.40	1.11
				Group II—Disease States							
7	J. B.	31	F	Psychoneurosis	66.6	161	38.4	39.2	1.34	1.04	1.29
8	M. W.	41	F	Cor pulmonale	49.0	152	46.0	46.0	1.87	1.75	1.07
9	J. C.	23	F	Cirrhosis	58.3	171	36.0	36.0	1.25	1.15	1.09
10	B. G.	39	F	Mitral stenosis	39.4	155	42.7	42.7	1.70	1.31	1.30
11	B. H.	54	F	Glomerulonephritis	73.0	161	39.7	39.7	1.62	1.47	1.10
12	J. N.	32	M	Constrictive pericarditis postop.	76.8	176	41.6	41.8	2.75	2.60	1.06
13	A. D.	31	F	Atrial sept. defect	45.0	161	47.3	46.7	2.43	2.10	1.16
14	W. K.	21	M	Hemangiomatosis	54.0	161	40.4	40.1	2.26	1.94	1.16
15	B. T.	68	M	Polycythemia vera	89.3	180	48.5	49.6	3.15	3.35	0.94
16	O. S.	19	M	Constrictive pericarditis postop.	78.0	183	45.8	46.0	2.82	2.40	1.17
17	J. P.	23	M	Congen. heart dis.	52.0	164	43.9	43.4	1.89	1.64	1.15
18	T. G.	39	M	Bronchial asthma	59.0	175	41.6	41.9	1.93	1.80	1.07
19	G. M.	43	F	Mitral stenosis postop.	60.5	157	40.7	40.7	1.64	1.47	1.12
20	R. J.	45	F	Polycythemia vera	55.0	166	60.0	60.0	2.94	2.56	1.15
21	G. D.	47	M	Rheum. heart dis.—failure	75.5	176	41.2	41.2	2.96	2.30	1.29
22	W. M.	35	M	Constrictive pericarditis postop.	74.4	181	48.0	48.0	3.24	2.69	1.20
23	M. S.	65	F	Osteitis deformans	65.5	169	44.8	43.7	2.10	1.75	1.20
24	E. S.	63	M	Cor pulmonale	65.9	165	63.5	63.5	4.90	3.70	1.32
25	A. B.	47	F	Patent ductus arteriosus	54.7	164	41.3	40.6	2.13	1.96	1.09
26	L. T.	44	F	Nephrotic syndrome	68.3	169	27.3	28.0	1.01	0.85	1.19
27	J. S.	29	F	Atrial sept. defect	46.2	168	40.8	40.9	2.33	1.86	1.25
28	L. J.	14	M	Nephrotic syndrome	59.9	177	32.0	31.9	1.55	1.35	1.15
29	M. B.	67	F	Rheum. heart dis.—failure	57.9	166	40.7	43.9	2.60	2.84	0.92
30	D. H.	25	M	Glomerulonephritis	97.3	181	44.9	44.9	3.88	3.01	1.29
31	C. S.	15	F	Patent ductus arteriosus	56.5	168	39.5	39.5	1.48	1.28	1.16
32	C. W.	49	M	Rheum. heart dis.—failure	82.8	191	40.7	40.8	3.42	3.20	1.07
33	M. P.	58	M	Polycythemia vera	88.8	171	59.4	59.9	4.43	3.85	1.15
34	G. C.	24	M	Tetralogy of Fallot	52.5	178	57.4	57.4	3.54	2.71	1.31
35	M. B.	67	F	Rheum. heart dis.—compensated	60.0	164	36.7	36.9	2.42	2.18	1.11

Mean ratio for Group I = 1.21 (S.D. 0.11)
Mean ratio for Group II = 1.15 (S.D. 0.10)
Mean of ratios for Group I and Group II combined = 1.16 (S.D. 0.10)

* Ratio of red blood cell volume as determined by CO (column 9) to red blood cell volume as determined by Cr^{51} (column 10).

The tube of blood was gently rotated for 40 minutes to insure fixation of the chromium in the cells. After the blood was centrifuged for 5 minutes at 3000 rpm, the supernatant plasma was removed. The cells were washed three times with Ringer's solution, suspended in the same solution to give the original volume of 15 ml., and refrigerated overnight at 4 to 5° C. Aseptic precautions were maintained throughout the *in vitro* handling of the blood.

By means of a small intravenous drip system, 10 ml. of the labeled cell suspension was injected from a cali-

tion. The volume of the solution used ranged between 0.2 and 0.5 ml. The scintillation counter had an efficiency such that 8 per cent of the gamma rays emitted from the Cr^{51} were registered as counts.

brated syringe and washed quantitatively into the subject's vein with not more than 30 ml. of normal saline solution. This amount of cell suspension gave a total of 12,000 to 20,000 counts per second in most cases. The indwelling needle was kept patent by an infusion of saline (8 to 10 drops per minute) until the required number of blood samples had been drawn. Samples for analysis were taken 10, 20, 30, and 45 minutes after the injection. In some experiments, an additional sample was taken at 24 hours. Prior to taking each sample, the first 3 ml. of blood withdrawn from the needle was discarded. The validity of taking the samples from the same needle and vein into which the tagged cells had been delivered was confirmed by finding, on several occasions, that results were essentially identical when samples were taken simultaneously from the indwelling needle and from a needle inserted in a vein of the opposite arm.

The Cr^{51} present in each sample was measured with a scintillation counter, using 2 ml. of whole blood in a dish 42 mm. in diameter. The same volume of a 1:50 dilution of the originally tagged cell suspension was used as a standard of comparison. A total of 4096 counts was made on each specimen. The counting error due to statistical fluctuations fell within ± 1.6 per cent.

The hematocrit of each sample of blood analyzed for CO or Cr^{51} content was determined in duplicate by the Wintrobe method. Tubes were centrifuged for 30 minutes at 3000 rpm at a radius of 15 cm.; no correction was made for trapping of plasma.

The total CO-available volume was calculated by the formula:

$$\text{CO space (liters)} = \frac{V_d \times P}{10(C_f - C_i)},$$

where V_d is the volume (ml.) of gas delivered into the rebreathing system, corrected to standard conditions of temperature and pressure, and P is a correction factor for purity of the gas. C_i and C_f, respectively, are the concentrations of CO (in ml. per 100 ml.) in samples of whole venous blood taken before and 20 minutes after delivery of the gas.

The total Cr^{51}-available volume was calculated similarly:

$$Cr^{51}\text{ space (liters)} = \frac{A_{tcs} \times 50 \times V_{tcs}}{1000 \times A_b},$$

where A_{tcs} is the activity in counts per second per ml. of the tagged cell suspension, V_{tcs} is the volume of cell suspension injected, and A_b the counts per second per ml. of the blood samples drawn at the end of the various time periods after delivery of the tagged cells.

The red cell volume (subsequently referred to as Vol. RBC/CO and Vol. RBC/Cr^{51}) was obtained by multiplying CO- or Cr^{51}-available space by the hematocrit of the respective sample of whole blood used in each analysis.

RESULTS

The results are summarized in Table I and Figure 1. Column 9, Table I, lists the apparent red cell volumes as determined by the CO method, and column 10, the volumes based on the 20-minute samples in the Cr^{51} method. The corresponding hematocrits are shown in columns 7 and 8.

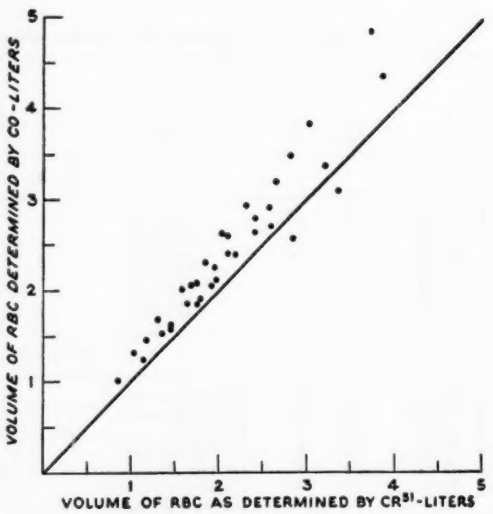

FIGURE 1.

Each point represents the apparent volume of RBC found by the CO method (CO-available space times the hematocrit of the sample analyzed for dilution of CO at 20 minutes), related to the apparent volume of RBC found by the Cr^{51} method (Cr^{51}-available space times the hematocrit of the sample analyzed for dilution of Cr^{51} at 20 minutes). The solid line represents a 1:1 relationship. (See Table I.)

As shown in Figure 1, Vol. RBC/CO was larger than Vol. RBC/Cr^{51} in both healthy subjects and patients with various diseases, with only two exceptions. The ratio of the CO to Cr^{51} values ranged from 0.92 to 1.32 for the entire series of 35 pairs of determinations. The mean ratio was 1.16, with a standard deviation of 0.10. In patients with a variety of pathologic states, the relationship between CO and Cr^{51} spaces was similar to that found in healthy subjects.

Considered in terms of absolute volumes, the average discrepancy of 16 per cent between the results of the two methods is of some magnitude. For example, the smallest and largest red cell volumes found by the chromium method were 0.85 and 3.85 liters. Assuming the average discrepancy of 16 per cent, these volumes would be 136 and 610 ml. greater, respectively, if determined

TABLE II

Red cell volume as determined by Cr^{51} and serial sampling

Subject*	Time of sample after injection of tagged cells					Per cent deviation between result of 24 hr. sample and 20 min. sample
	10 *min.*	20 *min.*	30 *min.*	45 *min.*	24 *hr.*	
1	—	1.58	1.69	1.69	1.76	+10.1
4	—	2.08	2.10	1.98	2.26	+ 8.0
5	—	1.97	2.01	2.01	2.10	+ 6.2
11	1.46	1.47	1.46	—	1.53	+ 3.9
15	3.30	3.47	3.51	3.47	3.36	− 3.2
18	1.93	1.90	—	1.97	1.94	+ 2.1
21	2.33	2.30	2.32	2.35	2.39	+ 3.9
22	2.63	2.69	2.83	2.70	2.92	+ 8.5

* Subjects are referred to by same numbers as in Table I.

by CO. Variations of this degree would be important in the application of these methods to physiologic and pathologic problems.

DISCUSSION

Whenever the measurement of blood volume or fractions thereof is based on the principle of dilution, two criteria must be satisfied. First, the substance which is introduced as indicator must have reached uniform concentration in the cell mass or plasma occupying all portions of the vascular compartment before the sample to be measured is taken. Second, the substance must not leave the blood stream during the mixing period which precedes the sampling. (In methods for determining "plasma" volume which are in use currently, the second criterion is not satisfied, but it is assumed that loss of the indicator occurs at a constant rate during the entire time required for mixing and for collection of serial samples.)

The first criterion probably is met adequately by both methods under consideration here. It has been demonstrated that with the method used in these experiments, CO gas becomes equilibrated with the blood in less than 20 minutes, and remains at essentially constant concentration in the blood for periods of an hour or more. This occurs with subjects at rest, and also during massive venous congestion of the extremities (15).

In our experience, maximum distribution of Cr^{51}-tagged red cells is also reached in 10 to 20 minutes in both normal and pathologic states, although it has been reported that in rare cases of severe congestive heart failure, longer periods may be required for complete mixing (21, 22). The results of serial sampling, which was done in each experiment reported here, showed no instance of delayed mixing in our subjects.

Table II and Figure 2 give the results of eight representative experiments in which samples were taken 10, 20, 30, and 45 minutes, and 24 hours after introduction of radioactively tagged cells. In every case the concentration of tagged cells in the blood had reached plateau levels within 20 minutes after the injection. Even after 24 hours, when some loss of tagged cells from the circulation was to be expected (5, 6, 22), the apparent increase in Vol. RBC/Cr^{51} averaged only 5 per cent and never was more than 10 per cent of the volume calculated from 20-minute samples. Thus, it appears unlikely that the discrepancy between results of red cell volume determinations with CO and Cr^{51}, when based on 20-minute mixing pe-

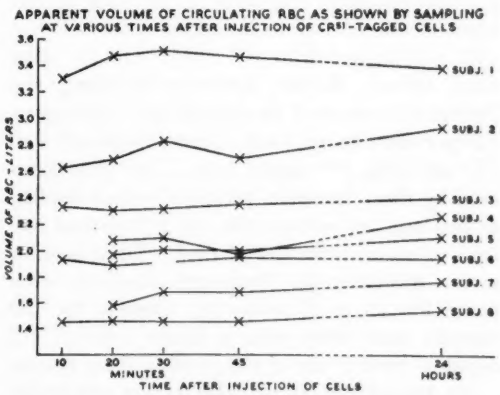

FIG. 2. THE APPARENT VOLUME OF RBC AS SHOWN BY ANALYSIS OF BLOOD SAMPLES OBTAINED AT 10, 20, 30, AND 45 MINUTES, AND AT 24 HOURS AFTER INTRAVENOUS INJECTION OF Cr^{51}-TAGGED CELLS, IN EIGHT HUMAN SUBJECTS. (SEE TABLE II.)

riods, can be attributed to delayed mixing of the chromium-tagged cells or to failure of these cells to reach portions of the circulatory system which were accessible to CO during the first 20 minutes after delivering either agent into the blood stream.

The discrepancy in results can be attributed more easily to failure of the CO method to fulfill the second criterion stated above. The finding of a CO-available space which is consistently larger than the space which is available to cells labeled with chromium might conceivably be attributed to 1) fixation of CO early in the mixing period by hemoglobin or other compounds which are located elsewhere than in the circulating blood, 2) metabolism or excretion of the CO, or 3) leakage of gas through faulty connections.

The first explanation is the most acceptable one. The characteristics of the time: concentration curve of CO in the blood are not consistent with continuing loss of CO by metabolism or excretion after the first 20 minutes of rebreathing (15). Care was taken to prevent loss of gas through leaks in the apparatus during each experiment. Furthermore, errors due to leakage would be expected to produce less consistent discrepancies between the results of the two methods than were actually found.

There is close quantitative agreement between these results in man and the observations of Root, Allen, and Gregersen in splenectomized dogs (18). These investigators found the red cell volume of the splenectomized dog to be 12 per cent greater when measured with cells tagged with CO than with cells tagged with P^{32} (18). On the other hand, Hevesy, Köster, Sørensen, Warburg, and Zerahn (9) reported an even greater discrepancy (24 per cent) between cell volumes measured with CO and with P^{32}-tagged cells. The procedures used by them, however, permitted errors tending to produce low values with the P^{32} method and high values with the CO method. These possible errors included: 1) Premature sampling when using P^{32} (5 to 10 minutes), whereas the CO samples were taken after a longer interval (15 minutes); and 2) use of a large rebreathing system.

No information is available as yet concerning the sites, presumably extravascular, where 12 to 16 per cent of the CO accumulates when introduced *via* the airways or by infusion of CO-tagged cells. According to Haldane and Smith (19), dissolved CO in plasma and body fluid can account for not over 0.2 per cent of all the CO distributed throughout the body. The fact that equilibrium is reached within 20 minutes after delivery of the gas, after which the concentration of CO in the blood remains nearly constant (15), suggests that the CO combines with compounds having an avidity for CO similar to that of hemoglobin. Experiments are in progress to determine the predominant sites of such combinations.

SUMMARY AND CONCLUSIONS

In 35 human subjects, 6 of whom were healthy and 29 suffering from a variety of diseases, simultaneous measurements were made of the apparent volume of red blood cells, using CO gas and cells tagged with Cr^{51}. The volume of distribution of CO 20 minutes after delivery of the gas was found to be consistently larger than that of the radioactively tagged cells at an equal interval after injection. The mean difference amounted to 16 per cent of the volume as measured with Cr^{51}-tagged cells. It is concluded that in this brief interval, significant quantities of CO leave the blood stream. The CO probably combines rapidly with compounds for which it has high affinity, but the sites where this occurs remain unknown.

Addendum: Recently we have found that better tagging of red cells with Cr^{51} is obtained when A.C.D. solution is used as anticoagulant instead of heparin during the *in vitro* handling of the blood.

ACKNOWLEDGMENT

We wish to acknowledge the able technical assistance of Mrs. Tillie Leake, Miss Vivian Ashford, and Miss Barbara Bradley.

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CLINICAL EFFECTS OF THE DICHLORO AND MONOCHLORO-PHENYL ANALOGUES OF DIAMINO PYRIMIDINE: ANTAGONISTS OF FOLIC ACID [1, 2]

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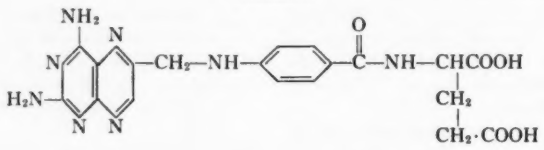
Hitchings, Elion, Vanderwerff, and Falco (1) demonstrated that certain 2,4-diamino-pyrimidines inhibited the growth of *L. casei;* this inhibition could be reversed by folic acid and citrovorum factor. The effects of these active pyrimidines are thus similar to those of the 4 amino derivatives of folic acid. But in contrast to the folic acid antagonists, the active pyrimidines are not pterins, and it appears unlikely that a rearrangement or structural alteration to a pterin ring system can occur. It is of interest, however, that high antagonist activity is associated with the presence of 2 and 4 amino groups in the pyrimidine nucleus, and a weighty substituent in the 5 position, suggesting a definite similarity in molecular configuration to the folic acid antagonists (Figure 1).

FIGURE 1



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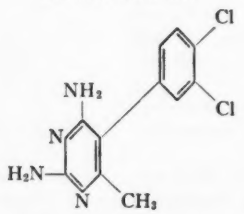

2,4-diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine (DDMP)

Because the 4-amino derivatives of folic acid inhibit the growth of tumors and leukemias in animals and in man (2), Clarke, Buckley, Sternberg, Stock, Rhoads, and Hitchings (3) tested a series of diamino-pyrimidines for their influence on transplantable mouse tumors, and observed that one of this group, 2,4-diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine (DDMP) inhibited the growth of the Sarcoma 180. The drug also produced inhibition in the growth of leukemia (4) and the Ehrlich ascites tumor (5) in mice, and modified the development of the chick embryo (6). At the doses tolerated under the conditions of testing, DDMP was the pyrimidine analogue most effective in inhibiting tumor growth. Since the folic acid antagonists are particularly effective

[1] This investigation was supported by research grants from the American Cancer Society, the Damon Runyon Memorial Fund for Cancer Research, the Lasker Foundation and the Grant Foundation, and by Grant C-1889 from the National Cancer Institute of the National Institutes of Health of the United States Public Health Service.

[2] We wish to express our appreciation to Dr. George H. Hitchings of the Wellcome Research Laboratories for the supply of DDMP and the Lederle Laboratories Division of the American Cyanamid Company for the supply of Leucovorin.

against acute leukemia in children, DDMP was examined for its therapeutic activity in this disease. In order to conduct a proper clinical assessment, it was also necessary to obtain information on the clinical pharmacology of DDMP, its activity in relation to the folic acid antagonists such as A-methopterin, and the protective action of Leucovorin.[3]

PHARMACOLOGY IN LABORATORY ANIMALS

The LD_{50} doses of DDMP by single intraperitoneal injection in mice and rats are 38 and 28 mg. per kg., respectively, and deaths usually are delayed, occurring within 3 to 10 days. Doses in excess of the LD_{50} cause an acute convulsive death. At the LD_{50} dose level, the delayed deaths can be prevented by the simultaneous administration of Leucovorin but not by folic acid. Leucovorin will not prevent the lethal effects of larger single doses of DDMP.

[3] Leucovorin, 5, 6, 7, 8 tetrahydro-5-formyl pteroylglutamic acid, is a synthetic product which has been shown to have one-half the microbiological activity of natural citrovorum factor (7).

The bone marrow of dogs receiving toxic doses of DDMP (1 mg. per kg. daily × 5) showed the megaloblastosis, giant metamyelocytes and hypersegmentation of the nucleus of the granulocytes (8, 9) seen with the 4-amino derivatives of folic acid.

CLINICAL RESULTS

Clinical toxicity

DDMP was administered orally to 44 patients. These patients were examined daily, and had daily hemoglobin and leukocyte determinations and weekly platelet and reticulocyte counts. In most cases the drug was given daily, but when early signs of oral or gastrointestinal toxicity or of leukocyte or platelet depression appeared, the drug was withheld temporarily. Thirty-four patients had an adequate trial of the drug, in that it was given until either signs of toxicity or a therapeutic response occurred.[4]

[4] The diagnoses and the number of adequately treated cases were: Acute leukemia, children, 19, adults, 6; single cases of rhabdomyosarcoma, chronic myelocytic leukemia, fibrosarcoma, reticulum cell sarcoma, melanoma, carcinomas of the adrenal, pancreas, rectum, lung and carcinoid of the colon.

TABLE I

Dosages in patients receiving adequate trial of DDMP

	No. of patients	No. courses of DDMP	Usual daily dose, *mg.*	Duration treatment, *days* Range	Duration treatment, *days* Aver.	Total dose, *mg.* Range	Total dose, *mg.* Aver.
Previously untreated with chemotherapy, or none within two weeks of DDMP							
Children							
Acute leukemia							
Previously untreated	9	13	2.5–5	10–33	22	40–145	78
Last dose of Amethopterin more than 2 weeks before	4	4	2.5–10	21–41	29	53–132	95
Adults							
Acute leukemia	4	6	5–10	4–39	24	35–175	108
Misc. cancers	8	8	2.5–20	8–68	33	50–635	280
Amethopterin given less than two weeks before or simultaneously with DDMP							
Children							
Acute leukemia	6	6	2.5–5	5–35	15	13–93	33
Adults							
Acute leukemia	2	4	2.5–15	4–25	13	30–163	66
Misc. cancers	2*	4	2.5–10	2–12	7	5–120	61

* These patients were also treated with DDMP alone.

The tolerated daily dose of DDMP ranged from 2.5 to 10 mg. in children and 2.5 to 20 mg. in adults (Table I). In the patients with acute leukemia, children could tolerate almost the same total dose as the adults. Many of the adults with lymphoma or carcinoma, however, tolerated larger daily and larger total doses than did the patients with leukemia. It was not possible to correlate satisfactorily, however, the absence of marrow involvement by neoplastic disease with the ability to tolerate larger doses.

A patient who received A-methopterin (2.5 mg.) together with the daily dose of DDMP (Figure 2) and the children who had received A-methopterin within two weeks prior to the onset of DDMP therapy, tolerated smaller amounts of the latter, and signs of toxicity appeared early in the course of treatment.

Toxic manifestations appeared in the gastrointestinal tract, the skin, and the hematopoietic system. Injury to the gastrointestinal tract was evidenced by nausea and vomiting, mouth ulcerations, or diarrhea (occasionally hemorrhagic). A morbiliform, erythematous, or follicular rash was noted frequently. Two adults, after prolonged therapy with DDMP, developed symmetrical, diffuse, brown pigmentation. This was most marked on the extensor surfaces of the joints of fingers and toes, and on their faces. Those children developing toxic rashes had residual brownish pigmentation

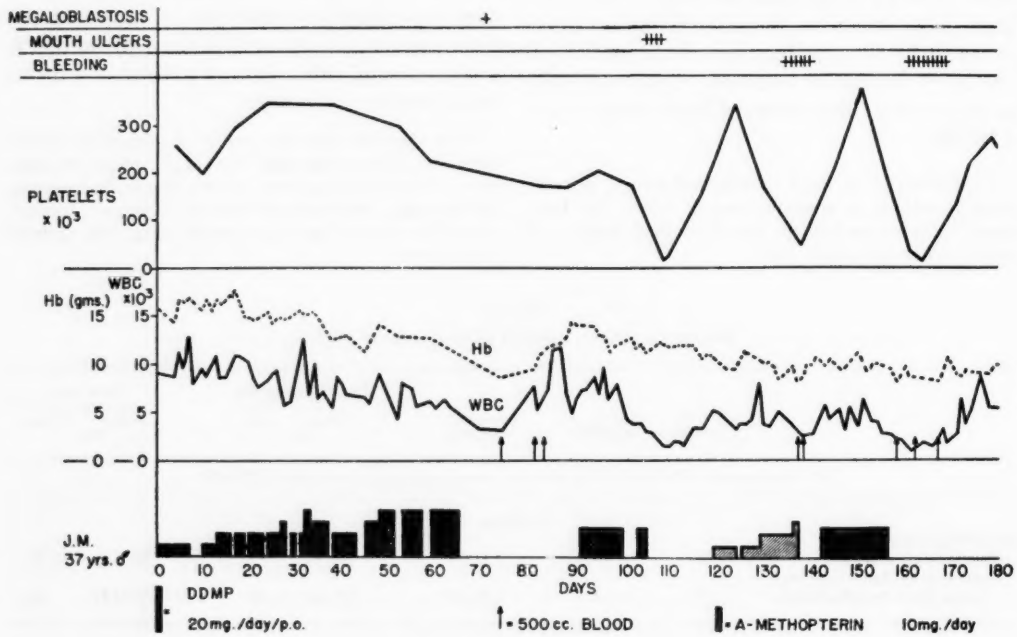

FIGURE 2

J. M., a 37-year-old colored man with carcinoma of the rectum, metastatic to the abdomen and chest, received a trial of DDMP to determine whether it had any effect on rectal cancer.To attempt to demonstrate a possible additive effect a combination of DDMP and A-methopterin was given. A total dose of 635 mg. of DDMP over a period of 65 days caused hematological toxicity with hypocellularity and megaloblastosis of the bone marrow and the drug was stopped. The abnormal blood picture returned to normal within four weeks; thereafter DDMP 10 mg. and A-methopterin 2.5 mg. daily were given for a total of 12 doses when hematological toxicity again appeared and treatment was stopped. Three weeks later, after recovery from the toxicity, A-methopterin at a total dose of 105 mg. produced an abrupt fall in the leukocyte and platelet counts. On discontinuing the drug the blood picture returned to normal. Another trial of combined therapy (DDMP—120 mg. and A-methopterin—30 mg. total dose) produced a fall in the leukocyte and platelet counts and treatment was discontinued. During the study there was no evidence of tumor inhibition and, since the disease was progressing, the patient received radiotherapy with no improvement. The patient expired, six weeks after the last dose of DDMP and A-methopterin. Permission for autopsy was not obtained.

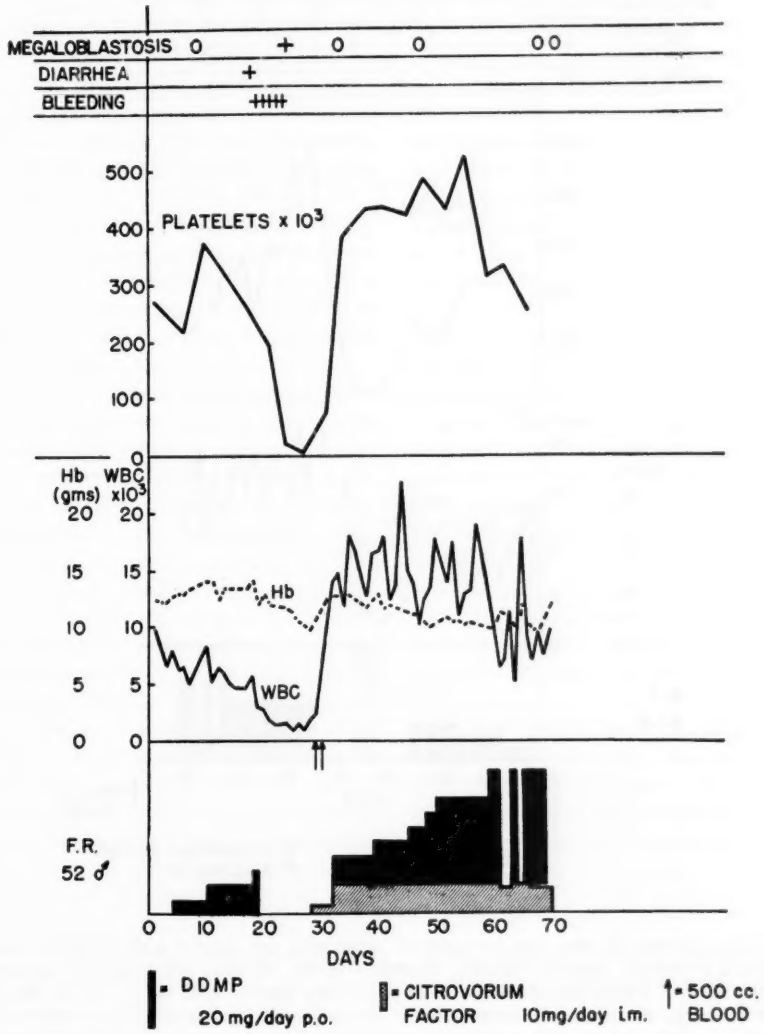

FIGURE 3

F. R., a 52-year-old man, with bronchogenic carcinoma, was treated with DDMP and toxic symptoms of leukopenia, thrombocytopenia, bleeding and rash occurred after a total dose of 125 mg. in 15 days (daily dose 5 to 15 mg.). There was no evidence of tumor regression. After the drug was stopped the blood picture improved. The patient was then restarted on DDMP together with Leucovorin. Despite increasingly large doses of DDMP (a total of 820 mg. in 38 days (10 to 40 mg. daily)), 10 mg. of Leucovorin daily protected against its hematological toxicity. Tumor regression did not occur on this regimen, and the patient expired. Permission for autopsy was not granted.

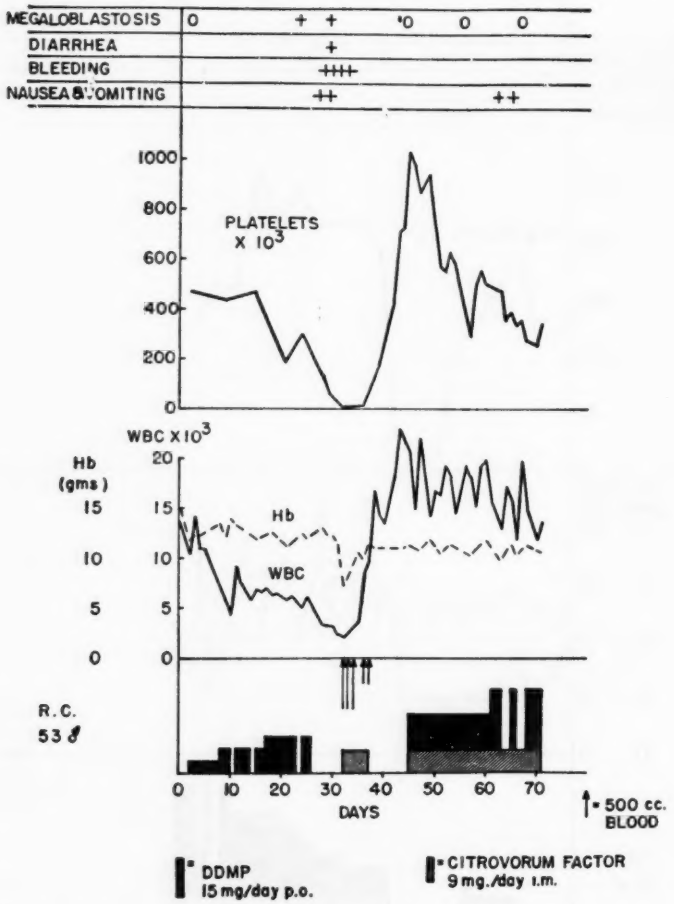

FIGURE 4

R. C., a 53-year-old man with bronchogenic carcinoma, was treated with DDMP. Toxic symptoms of nausea, vomiting, diarrhea, thrombocytopenia, bleeding, and leukopenia occurred after he received a total dose of 220 mg. in 24 days (daily dose: 5 to 15 mg.). At the time of greatest toxicity an increase in transmission of breath sounds in a previously atelectatic area was found. This probably did not indicate tumor regression, since cavitation of the tumor occurred subsequently. Hematologic findings improved when DDMP was discontinued. The patient subsequently received 415 mg. of DDMP in 26 days (15 to 25 mg. daily) simultaneously with Leucovorin,[5] without evidence of hematological toxicity, although nausea recurred.

Tumor progression continued, and the patient expired following a massive hemoptysis. Autopsy showed bronchogenic carcinoma of the right upper lobe with metastases to lymph nodes, right lower lobe, left lung, liver, right adrenal gland and bone marrow. There was cavitation of the primary tumor with massive hemorrhage and aspiration of blood into the right lower lobe and all lobes of the left lung.

[5] Microbiologic assay by Dr. Dorris Hutchison of the Leucovorin samples available for clinical use showed that there was only 10 to 20 per cent of the stated activity, so that the patient, R. C., was actually receiving 0.9 to 1.8 mg. of microbiologically active Leucovorin daily. Patient F. R. (Figure 3) had been studied 10 months earlier, and while the Leucovorin was not assayed microbiologically at that time, it is likely that this fresher preparation more closely approximated the stated activity.

at the site of the rash for as long as six months. Two children and two adults who received DDMP to the point of toxicity then developed alopecia. Skin pigmentation has been reported as occurring after prolonged administration of A-methopterin (10) and alopecia also is a previously noted complication of therapy with the 4-amino antagonists of folic acid (2).

A fall in the total leukocyte count to severely leukopenic levels frequently accompanied the other toxic manifestations. A decrease in the number of circulating platelets often occurred even in those patients whose bone marrow was essentially normal before therapy.

The bone marrow showed megaloblasts, differing slightly from those seen in pernicious anemia in that the nuclear chromatin was somewhat more granular, in 17 of the 34 patients receiving an adequate course of DDMP. Three showed normoblastic erythropoiesis. The marrow of the 14 remaining patients failed to show nucleated erythroid cells either due to the leukemic state or because, after therapy, inadequate specimens were obtained.

Giant metamyelocytes and hypersegmented polymorphonuclear leukocytes also were seen.

With certain differences, the pattern of toxicity observed resembles that seen after A-methopterin administration. Mouth ulcerations occurred in less than half of the patients adequately treated with DDMP and the ulcerations were more diffuse with generalized mucosal erythema. They usually appeared after the onset of leukopenia, rather than preceding it, as with A-methopterin, and were, therefore, not a useful premonitory sign of toxicity. Another factor making the drug more difficult to use than A-methopterin was the occasional progression of leukopenia, mouth ulcerations and gastrointestinal symptoms for as long as 7 to 10 days after DDMP was discontinued.

Evidence was obtained in two patients that the hematological toxicity of DDMP was prevented by the simultaneous administration of Leucovorin (Figures 3, 4).

Therapeutic trials

Of the 34 patients receiving an adequate trial of DDMP, a therapeutic response occurred only in children with acute leukemia. Of twelve children with acute leukemia treated initially with DDMP, three showed hematological and clinical improvement following, and presumably due to, the drug. Two of these remissions were complete, and lasted two and seven months, respectively. Hematological remission consisted of improvement in bone marrow with a decrease of the total of stem cells and lymphocytes to less than 30 per cent as well as a return of the total leukocyte and differential counts toward normal levels. The third child had a good clinical remission, but the differential count of the bone marrow smear showed a decrease of the stem cells from 98 to 55 per cent, with an increase in the erythroid precursors from 0 to 30 per cent. The drug had to be discontinued because the patient left the hospital, and it was considered too hazardous to continue on an out-patient basis.

Seven children who had been treated with A-methopterin prior to DDMP, and had developed resistance or were initially resistant to A-methopterin, showed no definite therapeutic response to an adequate trial of DDMP.

A 3-year-old child with embryonal rhabdomyosarcoma received an amount of the drug (DDMP) sufficient to cause megaloblastosis and depression of leukocytes and platelets. The tumor became necrotic but there was no significant change in size.

Fourteen adults received adequate courses of DDMP; six had acute leukemia and eight had other neoplastic diseases. Twelve showed significant leukocyte depressions, all developed other evidences of toxicity, and none showed a therapeutic response.

Comparison with Daraprim®

The phenyl-substituted members of the 2,4-diaminopyrimidine series have antimalarial activity, (11) and one of this group, 2,4-diamino-5-p-chlorophenyl-6-ethylpyrimidine (Daraprim®) has been used successfully in the suppression and prophylaxis of malaria in man (12–15). While the dichloro derivative, DDMP, is almost as active an antimalarial as the monochloro compound, Daraprim,® it has not been used because of its greater toxicity in man.

Daraprim® is also less toxic for animals (16) but, at tolerated doses in mice, no anti-tumor effect has been noted. Mice can be protected against the acute lethal convulsive seizures produced by Daraprim® by pre-treatment with sodium phenobarbital, so that larger doses can be given which

finally result in delayed deaths similar to those seen after DDMP. These larger doses did not inhibit the Sarcoma 180 (17).

Daraprim® appears to act as an antimetabolite. Hitchings and his co-workers have produced with Daraprim® (18) a syndrome resembling folic acid deficiency in the growing rat. The debilitation, hemosiderin deposits, alopecia, and leukocyte depression can be reversed by Leucovorin but not by folic acid. Liver powder and autolyzed yeast have even greater protective action than that expected on the basis of their citrovorum factor content. Whereas single doses of DDMP can produce this deficiency picture in rats, Daraprim® requires a high level and continuous administration. Daraprim® also produces megaloblastosis in dog bone marrow (8, 9).

The recommended dose of Daraprim® for suppression or prophylaxis of human malaria is 25 mg. weekly. This compound has been administered at 5 mg. daily for one year, 50 mg. twice weekly for three months (13, 14) and 25 mg. daily for 50 consecutive days (15) with no evidence of toxicity other than a transient fall in hemoglobin level and erythrocyte count in one patient.

In our studies, two patients were treated with Daraprim® at doses higher than those used in antimalarial therapy; no therapeutic effect on the neoplastic disease was observed. The toxic effects, other than nausea and vomiting, were hematologic, with platelet and leukocyte depressions, anemia, and megaloblastosis. One patient with a metastatic synovioma was given a total of 1500 mg. of Daraprim® in a 26-day period, the daily dose ranging from 50 to 100 mg. One week after therapy was stopped the platelet count fell from 250,000 to 27,000 per cu. mm. with a slight fall in hemoglobin level, but the leukocyte count remained unchanged. The platelet count remained low for three days and then rose to normal levels.

The second patient was a 49-year-old woman with widely disseminated metastatic carcinoma, probably primary in the uterus. She was markedly cachectic, had evidence of liver metastases, and a normal blood urea nitrogen. She received three daily doses of 100 mg. each of Daraprim® and the drug was then discontinued because of severe nausea and vomiting. Five days later the leukocyte count fell from the pretreatment level of 18,000 to 1,200 per cu. mm. The sternal marrow aspirate, at this time, was cellular with definite megaloblastosis, giant metamyelocytes, and an increase in plasma cells. The leukocyte count continued to fall to 500 per cu. mm., and thrombocytopenia and bleeding tendencies developed. The bone marrow reverted to a normoblastic picture, but the platelet count remained low, bleeding continued and, despite repeated blood transfusions, the patient died 18 days after the last dose of Daraprim®. Permission for autopsy was not obtained.

It is possible that the inadequate diet of this patient prior to Daraprim® therapy, as well as the pre-existent liver damage, may have increased her sensitivity to the antimetabolite, possibly because of a borderline folic acid deficiency.

DISCUSSION

It has been postulated that folic acid is essential for cellular growth and division because of its role in purine and pyrimidine metabolism. Folic acid has been implicated in the activation of such reactions as the formation of amino acids, transfer of methyl groups, methylation of purine ribosides, and closure of the purine ring. In order to carry out its functions, folic acid is converted to its effective form, the citrovorum factor. The conversion of folic acid to citrovorum factor is blocked by the 4-amino derivatives of folic acid, which explains the poor protection provided by folic acid in animals.

The available evidence indicates that DDMP, while structurally quite different, acts by interfering with the same metabolic pathways as those affected by A-methopterin, although it may act at somewhat different loci. Experimental work suggests that the 2,4-diaminopyrimidines interfere both with the conversion of folic acid to citrovorum factor and with its utilization or further transformations.

DDMP and Daraprim®, like A-methopterin, produce megaloblasts, giant metamyelocytes and hypersegmented polymorphonuclear leukocytes in the bone marrow. The diaminopyrimidines inhibit the growth of *L. casei* (1), and this is reversed competitively by folic acid. Folic acid, however, cannot reverse or prevent the inhibition of growth caused by these 2,4-diaminopyrimidines in *S. faecalis* (18) or in intact animals (19). The inhibition of bacterial growth can be prevented

competitively by Leucovorin in *L. citrovorum* and over a narrow range of concentration in *S. faecalis* (18). In mice and rats, however, although Leucovorin can prevent the lethal effects of DDMP when one to three times the LD_{50} dose is given, when the dose of the antimetabolite is raised significantly above this level, increasing the amount of Leucovorin does not prevent death. This differs from the protective effect afforded by Leucovorin against A-methopterin in mice (20). In patients, Leucovorin protected against the toxicity of DDMP, in that three to six times the total amount of the drug, which had previously produced hematologic and clinical signs of toxicity, had no toxic effect when administered simultaneously with much smaller doses of Leucovorin.

Despite the similarity in some of the effects of DDMP and A-methopterin, there are clinical differences, suggesting that these agents may have different sites of action on the metabolic pathways involving folic acid and citrovorum factor. Toxic manifestations persist longer after the discontinuation of DDMP therapy; mouth ulceration may develop concurrently with diarrhea and leukocyte depression rather than preceding them, or may be absent entirely. Thus it is not as satisfactory a warning sign as with A-methopterin. The leukocyte depression tends to be more delayed and protracted. A possible explanation is that, after an ingested dose of A-methopterin, serum levels fall and are no longer detectable in about 3 to 8 hours, and approximately 40 to 60 per cent of the dose is excreted within 12 hours (21); whereas there is indirect evidence based on Daraprim® studies (22) suggesting that DDMP may disappear much more slowly. The postulated prolonged maintenance of serum levels of DDMP does not necessarily explain this difference in toxicity, however, since A-methopterin has been demonstrated in measurable amounts in an unchanged form (as shown by microbiological assay and paper chromatography) in mouse liver at least eight months after the administration of this compound (23). The tendency of A-methopterin to persist in the tissues may account for the synergistic effect of A-methopterin and DDMP.

DDMP may be expected to produce transient hematological remissions in about one-fourth of the children with acute leukemia, and its therapeutic activity thus parallels, to some extent, that of A-methopterin. It was not therapeutically effective in children who were resistant, or who had responded and then become resistant, to A-methopterin, and its greater toxicity and difficulty in safely controlling dosage prevents it from being a useful substitute for for A-methopterin.

SUMMARY

1. The compound 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine, DDMP, is an antagonist of the folic acid system although it is structurally quite different. In man it produces toxic effects similar to the 4-amino analogues of folic acid. These effects are principally bone marrow depression with megaloblastosis, mouth ulcerations, digestive disturbances and rash. DDMP differs from A-methopterin, however, in that it appears to have a more prolonged toxic effect, and mouth lesions are less conspicuous and less frequent. DDMP produced hematological improvement in 3 of 12 children with acute leukemia, but it was ineffective in children resistant to A-methopterin. Leucovorin protected against large doses of DDMP in two patients. A-methopterin and DDMP were found to have additive toxic effects, and DDMP was more toxic in children who had received A-methopterin within two weeks prior to DDMP therapy.

2. The antimalarial, Daraprim® (2,4-diamino-5-p-chlorophenyl-6-ethylpyrimidine), which is structurally related to DDMP, produced simliar toxicological effects in two patients when given in large doses by weight.

3. The clinical data support the view that a pyrimidine, DDMP, acts as an antimetabolite in the folic acid-citrovorum factor system. While DDMP may act in inhibiting the transformation of folic acid to citrovorum factor, as do the 4-amino derivatives of folic acid such as A-methopterin, its loci of action are not entirely the same.

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METABOLIC EFFECTS OF MARKED SODIUM RESTRICTION IN HYPERTENSIVE PATIENTS: CHANGES IN TOTAL EXCHANGEABLE SODIUM AND POTASSIUM [1]

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It is important to understand the mechanism by which drastic sodium restriction effects a decrease in blood pressure in some patients with essential hypertension. It has been shown (1) that a decline in total exchangeable sodium (TENa) as measured with the isotope Na^{24}, is not correlated with a decline in blood pressure, and therefore simple "desalting" of the body can not account for the phenomenon. The important relationship which exists between sodium and potassium in most living species, both animal and plant, suggested the possibility that sodium restriction might be effective in lowering blood pressure through secondary effects on K. With the isotopes Na^{24} and K^{42}, nearly all of the body's Na and K pools can be measured (2, 3). Therefore, it is possible to quantitate accurately the effects of marked Na restriction upon these pools and to determine whether the effects of Na restriction on total exchangeable potassium (TEK) may be correlated with alterations in blood pressure. The present paper is a report of a study on 11 patients with essential hypertension who were given a basic low Na diet supplemented during the period of control observations with 10 grams (180 mEq.) of enteric coated NaCl per day. The removal of this added NaCl during the subsequent test period constituted the sole change in regimen. During both phases of the study after suitable periods for equilibration the effects on TENa, TEK, and blood pressure were studied.

PROCEDURE

Patients and clinical status

Eleven adult patients with essential hypertension were studied for a 3-month period on the metabolic wards of the Brookhaven National Laboratory Hospital. These people were admitted in two groups; one consisted of two males and four females from April to July (Group I), the other of three males and two females from September to December (Group II), 1953. Since the primary purpose of this communication is not concerned with the clinical effects of sodium restriction which have been reported frequently in the past (4–6) itemized details of each patient's status relative to laboratory findings have been omitted from this report except as seemed pertinent. With the two exceptions noted below (patients M and R) all patients were of the type classed as benign essential hypertension. Urea clearances were normal in all, but variable degrees of albuminuria were present in some patients. Intravenous pyelograms were normal except in patient R as noted. Serum cholesterol, CO_2, Cl, total protein, albumin and globulin, sodium and potassium were within normal limits in all patients. Electrocardiograms ranged from normal to variable degrees of left axis deviation and low or inverted T waves. (Tracings on patient R revealed classical evidence of acute myocardial infarction involving the anterior wall of the left ventricle during the two episodes described subsequently.) Heart size was shown by X-ray to vary from normal in some to moderate, diffuse enlargement in others. None gave past histories which suggested episodes of acute heart failure. Benzodioxane tests for pheochromocytoma were negative in all. Both males mentioned earlier had serious disease; M, age 53, had had a right hemiplegia six months prior to admission with residual weakness and spasticity. Upon admission, he was found to have mild diabetes mellitus as well, which was readily controlled by small doses of protamine zinc insulin. During his hospital stay, he had two transient cerebrovascular thromboses, from both of which return to previous level of function occurred in three to seven days. This patient's disease was obviously severe and about six weeks after his initial discharge he died from multiple cerebrovascular thromboses. Patient R, age 47, who had had angina on effort for several years, developed two mild coronary occlusions five and seven weeks after admission with uneventful recovery. He had had a chronic duodenal ulcer of more than 20 years duration which plagued both patient and staff during the study. By intravenous pyelogram he was shown to have a small asymptomatic calculus in each kidney, measuring 5 and 15 mm. in diameter, respectively; these were presumed to be secondary to the heavy alkali and milk ingestion for many years.

Experimental program

The patients remained ambulatory throughout their stay except during studies or intercurrent illnesses. Upon

[1] This work was carried out under the auspices of the Atomic Energy Commission.

admission, each patient was placed on a basic diet calculated to yield about 6 mEq. sodium per day (analysis of 18 separate diets yielded a mean value of 5.7 mEq. with S.D. ± 1.4); because of this restriction the daily protein intake averaged approximately 55 grams. In Group I, protein supplement, although allowed, was not required to be eaten; associated with this, members of the group lost variable and often significant amounts of weight. Since previous work (7) had shown that the levels of protein in the diet had no effect upon blood pressure, weight loss seemed unimportant. In view of the changes in TENa and TEK, subsequently calculated to be present, it was thought necessary in the future to control the factor of weight loss as well. Therefore, members of the second group were required to consume a total of 1.5 grams of protein per Kg. per day through low Na protein supplements with Lesofac[2] or Lonalac®; on this regimen, the weights remained within narrow limits, with the maximal change recorded being a gain of 4.0 Kg. in patient B. Salt-free or salt-poor carbohydrates and fats were given in amounts sufficient to satisfy caloric needs. The diets were calculated to contain 30 to 100 mEq. of potassium per day and this was confirmed by analyses. The first five to six weeks served as the control period; therefore, during these first weeks 10 grams (180 mEq.) of sodium chloride in enteric coated capsules were administered daily to each patient in divided doses, an amount which is regarded as being within the average of American intakes (8). At the termination of the control observations, the sole change in the regimen was the omission of the added NaCl, so that the effects of this restriction alone could be studied. At intervals before and after this sharp decrease in NaCl intake, TENa and TEK were measured as well as other studies appropriate for evaluation of clinical status.

METHODS

Blood pressure, urine and blood collections; sodium and potassium analyses

Blood pressures were measured six mornings a week by one of the authors under standard conditions; namely, after breakfast with a minimum bed rest of half an hour before the readings were made. Systolic and diastolic pressures were recorded as the first appearance and total disappearance of sound respectively as recommended by a committee of the American Heart Association (9). All urines in each 24-hour period were pooled, the volumes measured, aliquots removed, and then preserved with 1 per cent HCl for subsequent chemical analysis of sodium and potassium with a Perkin-Elmer Model 52A flame photometer, with a lithium internal standard. The volume of the urine samples used for analysis ranged from 1 to 40 ml. depending upon the concentration of Na or K in the specimen. Blood samples were removed without stasis, mixed in heparin tubes and centrifuged within 5 to 10 minutes. With care in handling the samples visible hemolysis was not seen. From the plasma so obtained, 1 ml. samples were analyzed in duplicate with the flame photometer in the fashion used for urine.

[2] Obtained through the courtesy of Wyeth Laboratories, Philadelphia, Pennsylvania.

Total exchangeable sodium (TENa)

TENa was determined by an isotope dilution technique with the procedure essentially as described earlier (1). The isotope $Na^{24}CO_3$ was obtained from the Brookhaven National Laboratory reactor, sterilized, then prepared for administration by diluting with 5 per cent dextrose; of this each patient received about 100 μc (containing approximately .06 mEq. Na^{24}) intravenously from a calibrated 100 ml. buret over a 7- to 10-minute period. Plasma samples were always taken at 5, 7, and 24 hours in the first group and at 24 and 26 hours in the second group; the final 24- and 26-hour specimens were always collected with the patients in the fasting state. Thus, in Group I the value for final exchangeable sodium represented the corrected 24-hour figure and was in all instances a reasonable extension of the values found earlier. In Group II, the final figure was the mean of the 24- and 26-hour values at both of which times presumably the maximal value for TENa had been reached. Based on the data of Edelman, James, Baden, and Moore (2) it is highly improbable that a much longer period of equilibration would have affected the values for TENa obtained here. During the low sodium phase of these and related studies, correction for Na^{24} excretion in the urine was found to be negligible whereas during the high sodium phase the average 24-hour excretion was 6.3 per cent (range 3.2 to 10.0 per cent) of the injected dose.

Radioactivity was measured with a Texaco well-type Geiger counter and standard scaler; 5 ml. liquid samples were counted until at least 10,000 counts above background were recorded in duplicate on specimens of blood, urine, and standards. During the low sodium phase, however, urines were counted only long enough to demonstrate that the specific activity was not significantly above background. Correction for decay was made by counting at precisely spaced intervals *seriatim* Standard No. 1, Sample No. 1, Sample No. 2, Standard No. 2 with the counting intervals so arranged that the average of the times of counting samples and standards were at the same mid-point in time, with decay corrections having been made automatically thereby.

Total exchangeable potassium (TEK)

TEK was measured by an isotope dilution technique (3). Approximately 100 to 150 μc $K^{42}CO_3$ obtained from the Brookhaven reactor (containing about .04 mEq. K^{39}) were administered by the same technique used for estimation of exchangeable sodium. In Group I, all urines were pooled up to 37 hours with spot specimens collected after an overnight fast at 38, 40, and 42 hours, at which times probably 95 per cent of the total body potassium is in equilibration with K^{42} (3). The average specific activity of these three samples, corrected for loss of the isotope in urine, was used to calculate total exchangeable potassium. The average excretion of the administered K^{42} at 40 hours in 34 consecutive determinations was 5.5

per cent (range 1.2 to 8.7 per cent) and did not change during either phase of the present study. In Group II, the period allowed for equilibration was an average of 20 hours with all urine pooled up to 17 hours and spot samples collected under fasting conditions at 18, 20, and 22 hours. In no instance did it appear that equilibration had failed to occur in these times as manifested by a regular increase in TEK. If these 20-hour determinations underestimated the exchangeable K slightly relative to the 40-hour values (3), since each patient was his own control, the final relative values during high and low Na would not be affected. A cation exchange resin of the sulfonic type (Amberlite IR–120) was used to concentrate the urine potassium and thereby enhance the counting rate approximately eight-fold. Counting was done with a Harshaw scintillation crystal using a special input amplifier designed at the Brookhaven Laboratory and an Atomic Scaler (Model 1050A). Three ml. liquid samples of standard and sample were counted in the fashion described for Na^{24} using the same technique for decay correction.

Statistical analyses of significance

The total exchangeable sodium values were calculated from the following data: serum sodium concentration, the volume and counting rate of the isotopic solution administered with correction for the amount excreted where this was significant, and the counting rate of serum at the time of equilibration. Serum sodium determinations in duplicate were reproducible within 0.5 mEq., which amounts to less than a 0.4 per cent difference. Recovery experiments in which standard saline solutions were added to plasma gave maximal errors of 1 per cent; therefore, an error of 1 per cent has been allowed. The infusion fluid was administered in a 100 ml. buret, calibrated to contain 100.14 ml.; this correction has been disregarded. In this series, the average counting rate of infusion fluid and serum were 5000 and 2500 counts per minute (cpm), with total counts on duplicate samples averaging 27,500, and 25,000, respectively. The coefficient of variation calculated from standard deviation is respectively, .0063 and .0060 (10). Because of local conditions (both a reactor and 2.5 billion electron volt proton accelerator within 1.5 miles) background averaged 400 cpm, with a total of about 70,000 background counts per study. The coefficient of variation here amounts to .0040 and has been included in the calculation of total error. The final cumulative error has been calculated from the formula $\sigma^2 = \sqrt{\sigma_1^2 + \sigma_2^2}$ . . . (11) where σ equals cumula-

TABLE Ia

Data on Patients with Significant Declines in both Systolic and Diastolic Pressures

	Blood Pressure (Mean & S.D.)				Days before (-) or after (+) Na Restriction	Body Weight (Kg)			Exchangeable Sodium Data					Exchangeable Potassium Data				
											Difference from Preceding Study x					Difference from Preceding Study x		
Pt.	Syst.	p	Diast.	p		Admission	Study	Δ Admission	Total (meq)	Per Kg (meq/Kg)	Δ Days	Δ Wt. (kg)	Δ TENa (meq)	Total (meq)	Per Kg (meq/Kg)	Δ Days	Δ Wt. (Kg)	Δ TEK (meq)
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
C ♀ 39 (#5420)					-35	71.7												
					-28		70.5	-1.2	2531	35.9								
					-21		70.4	-1.3						2558	36.3			
	162		111		-12		69.7	-2.0						2391	34.3	9	-0.7	-167
	(10)		(9)		-8		69.7	-2.0	2443	35.1	20	-0.8	-88					
					+19		69.0	-2.7						2253	32.7	31	-0.7	-138
					+27		68.7	-3.0	2320	33.8	35	-1.0	-123					
I	130	<.01	94	<.01	+39		68.6	-3.1						2284	33.3	20	-0.4	+29
	(6)		(6)		+47		68.2	-3.5						2320	34.0	8	-0.4	+36
					+51		68.0	-3.7	2359	34.7	24	-0.7	+39					
A ♂ 42 (#5455)					-42	82.4												
					-19		81.4	-1.0	3127	38.4								
					-16		82.2	-0.2	3207	39.1	3	+0.8	+80					
					-12		81.4	-1.0						2879	35.4			
	159		97		-9		81.9	-0.5						2935	35.8	3	+0.5	+56
	(9)		(6)															
					+30		81.0	-1.4	2922	36.1	46	-1.2	-285					
					+37		81.3	-1.1						3248	40.0	46	-0.6	+313
					+40		81.5	-0.9	2922	36.1	10	+0.5	0					
II					+44		81.9	-0.5						3231	39.5	7	+0.6	-17
	133	<.01	85	<.01	+46		81.5	-0.9	2968	36.5	6	0.0	+46					
	(14)		(7)		+53		81.5	-0.9						3021	37.1	9	-0.4	-201
Mon ♂ 45 (#5472)					-42	83.1												
					-19		82.1	-1.0	3190	38.9								
					-16		82.2	-0.9	3075	37.4	3	+0.1	-115					
					-12		81.7	-1.4						2844	34.8			
	218		135		-9		81.9	-1.2						2860	34.9	3	+0.2	+16
	(14)		(8)															
					+30		82.5	-0.6	2974	36.0	46	+0.3	-101					
					+37		83.3	+0.2						3329	40.0	46	+1.4	+469
					+40		83.3	+0.2	3057	36.7	10	+0.8	+83					
II					+44		83.5	+0.4						3223	38.6	7	+0.2	-106
	199	<.01	124	<.01	+46		83.4	+0.3	2991	35.9	6	+0.1	-66					
	(15)		(8)		+53		83.9	+0.8						2962	35.3	9	+0.4	-261

Roman numerals I and II in column 1 refer to patient group I (April - July) or group II (Sept. - Dec. '53).
Mean blood pressures were calculated from the values measured during the last 10 days of control and low Na periods respectively.
Figures in parentheses are standard deviations from the mean.
p (column 3 and 5) = probability level calculated by Fishers "t-test" method.
Dark figures in column 10 and 15 represent values which differ from those preceding by more than 3 S.D.

x = Values are intervals (Δ) in days and changes (Δ) in weight and TENa or TEK respectively, found in the successive studies reported in this paper.

TABLE Ib

Data on Patients with Significant Decline in Only Systolic or Diastolic Pressures

	Blood Pressure (Mean & S.D.)				Days before (-) or after (+) Na Restriction	Body Weight (Kg)			Exchangeable Sodium Data					Exchangeable Potassium Data				
											Difference from Preceding Study x					Difference from Preceding Study x		
Pt.	Syst.	p	Diast.	p		Admission	Study	Δ Admission	Total (meq)	Per Kg (meq/Kg)	Δ Days	Δ Wt. (kg)	Δ TENa (meq)	Total (meq)	Per Kg (meq/Kg)	Δ Days	Δ Wt. (Kg)	Δ TEK (meq)
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
E ♀46 (#9274)	185 (10)		104 (7)		-35	56.8												
					-28		56.3	-0.5	1900	33.7								
					-21		55.8	-1.0						1867	33.5			
					-12		55.2	-1.6						1983	35.9	9	-0.6	+116
					-8		55.0	-1.8	1925	35.0	20	-1.3	+25					
I	157 (10)	<.01	98 (8)	<.2	+19		53.7	-3.1						1926	35.9	31	-1.5	+57
					+27		53.0	-3.8	1747	33.0	35	-2.0	-178					
					+39		53.2	-3.6						1807	34.0	20	-0.5	-119
					+47		52.4	-4.4						1793	34.2	8	-0.8	-14
					+51		52.7	-4.1	1801	34.2	24	-0.3	+54					
P ♀72 (#9424)	182 (17)		83 (6)		-37	58.1												
					-37		58.1	0.0	2288	39.4								
					-30		57.4	-0.7						1480	25.8			
					-21		57.3	-0.8						1605	28.0	9	-0.1	+125
					-16		57.2	-0.9	2366	41.4	21	-0.9	+78					
I	146 (11)	<.01	76 (7)	<.05	+10		53.3	-4.8						1496	28.1	31	-4.0	-109
					+19		53.2	-4.9	1977	37.1	36	-4.0	-389					
					+31		53.2	-4.9						1539	28.9	21	-0.1	+43
					+38		53.1	-5.0						1642	30.9	7	-0.1	+103
					+43		52.8	-5.3	2193	41.5	24	-0.4	+216					
					+75		53.6	-4.6	1957	36.5	32	+0.8	-236					
					+78		53.6	-4.5						1433	26.7	40	+0.5	-209
	165 (12)	<.01	80 (8)	<.4>.3	□ -18		54.6	-3.5	2287	41.9	22	+1.0	+330					
					-20		54.5	-3.6						1439	26.4	22	+0.9	+6
B ♀69 (#9504)	201 (14)		95 (11)		-42	62.2												
					-19		62.6//	+0.4	2331	37.2								
					-16		64.9	+2.7	2332	35.9	3	+2.3	+1					
					-12		65.3	+3.1						1833	28.1			
					-9		65.4	+3.2						1880	28.4	3	+0.1	+47
II	178 (11)	<.01	89 (9)	<.2>.1	+30		65.6	+3.4	2184	33.3	46	+0.7	-148					
					+37		65.8	+3.6						2023	30.8	46	+0.4	+143
					+40		65.7	+3.5	2232	33.9	10	+0.1	+48					
					+44		66.2	+4.0						1905	28.8	7	+0.4	-118
					+46		65.9	+3.7	2187	33.3	6	+0.2	-45					
					+53		65.9	+3.7						1812	27.5	9	-0.3	-93
L ♂42 (#9458)	172 (18)		101 (4)		-42	73.1												
					-19		72.8	-0.3	3002	41.2								
					-16		73.8	+0.7	3023	41.0	3	+1.0	+21					
					-12		72.8	-0.3						3057	42.0			
					-9		73.8	+0.7						3047	41.3	3	+1.0	-10
II	162 (15)	<.2>.1	83 (11)	<.01	+30		72.3	-0.8	2639	36.5	46	-1.5	-384					
					+37		72.0	-1.1						3259	45.3	46	-1.8	+212
					+40		71.7	-1.4	2708	37.8	10	-0.6	+69					
					+44		71.7	-1.4						3178	44.3	7	-0.3	-81
					+46		60.6	-2.5	2657	37.6	3	-1.1	-51					
					+53		71.6	-1.5						3063	42.8	9	-0.1	-115

See bottom of Table Ia for details.

□ = Days after Na added second time.

// = Weight may be erroneous since weights on days preceding and following were 64.5 and 64.9 Kg respectively.

tive error, and σ_1, σ_2 . . . etc. denote individual analytical errors. Using this formula, the average standard deviation of the entire procedure was .014, equivalent to about 40 mEq. for a 70-Kg. adult. This calculated value subsequently proved to be in substantial agreement with that found in 29 consecutive determinations on six patients in which the mean difference of duplicate values for TENa obtained at equilibrium, namely 24 and 26 hours, was 40.4 mEq., uncorrected for weight. The value for TEK was based on primary data similar to those used for sodium, but the standard deviation of the counts among the three urine samples has also been included, the value amounting to 3.8 per cent. Based on average rates of 10,000, 1440 and 220 cpm for infusion fluid, urine samples, and background total counts of 20,000 12,000 and 30,000, respectively, the coefficients of variance are .0092, .0070 and .0058. A value of .01 has been given to the chemical analysis for K on the basis of analytical data similar to those obtained for sodium. The cumulative error of these five values becomes .041, equivalent to about 80 mEq. for a 60-Kg. female (12) and 130 mEq. for a 70-Kg. male (3). Standard deviations of blood pressures were calculated from the mean of readings obtained during the final 10 days of both the high (control) and low (test) sodium phases. Fisher's "t" test was used as a criterion of significance between the mean values so found, with $p = < .01$.

OBSERVATIONS

The results of these studies are summarized in Tables Ia, Ib, and Ic in which the data are listed and divided among those subjects with a fall in

TABLE Ic

Data on Patients with Significant Decline in neither Systolic nor Diastolic Pressures

Pt.	Blood Pressure (Mean & S.D.) Syst.	p	Diast.	p	Days Before (-) or after (+) Na Restriction	Body Weight (Kg) Admission	Study	Δ Admission	Exchangeable Sodium Data Total (meq)	Per Kg. (meq/Kg)	Difference from Preceding Study x Δ Days	Δ Wt. (kg)	Δ TENa (meq)	Exchangeable Potassium Data Total (meq)	Per Kg (meq/Kg)	Difference from Preceding Study x Δ Days	Δ Wt. (Kg)	Δ TEK (meq)
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Mor ♂ 53 (#3283)	195 (9)		118 (10)		-35	64.1												
					-28		64.1	0.0	2681	41.8								
					-8		63.2	+0.9	2758	43.6	20	-0.9	+77					
					-2		63.5	-0.6						2280 | • 1784 |	35.9 28.1			
I					+19		62.1	-2.0						2049	33.0	21	-1.4	-231
					+27		63.0	-1.1	2606	41.4	35	-0.2	-152					
					+39		62.2	-1.9						2256	36.3	20	-0.8	+207
	192 (9)	<.5	115 (8)	<.5	+47		62.2	-1.9						2188	35.2	8	0.0	-68
					+51		61.8	-2.3	2671	43.2	24	-1.2	+65					
R ♂ 48 (#3303)	173 (14)		101 (6)		-35	92.7												
					-28		88.2	-4.5	3022	34.3								
					-21		87.4	-5.3						4011	45.9			
					-12		86.0	-6.7						4042	47.0	9	-1.4	+31
					-8		84.4	-8.3	2738	32.4	20	-3.8	-284					
I	165 (13)	<.3	98 (8)	<.4	+27		80.4	-12.3	2862	35.6	35	-4.0	+124					
					+39		82.4	-10.3						3505	42.5	51	-3.6	-537
					+47		81.2	-11.5						3554	43.8	8	-1.2	+49
					+51		80.6	-12.1	2992	37.1	24	+0.2	+130					
U ♀ 20 (#3348)	202 (13)		120 (8)		-35	44.4												
					-26		46.7	+2.3						2269	48.6			
					-22		45.7	+1.3	2180	47.7								
					-7		45.4	+1.0	2073	45.4	15	-0.3	-107					
					-1		44.7	+0.3						1633#	36.5	25	-2.0	-636
I	189 (13)	<.05	113 (9)	<.1	+13		44.6	+0.2	1956	43.9	20	-0.8	-117					
					+25		45.5	+1.1						2183	48.0	26	+0.8	-86◎
					+30		45.9	+1.5						2192	47.8	5	+0.4	+9
					+36		45.7	+1.3	2057	45.1	23	+1.1	+101					
S ♀ 53 (#6268)	196 (33)		90 (11)		-42	51.5												
					-19		51.6	+0.1	1997	38.3								
					-16		52.0	+0.5	1845	35.5	3	+0.4	-132					
					-12		52.2	+0.7						1631	31.3			
					-9		51.8	+0.3						1570	30.3	3	-0.4	-61
II	186 (19)	<.5>.4	88 (8)	<.5	+30		52.9	+1.4	1862	35.2	46	+0.9	+17					
					+37		52.2	+0.7						1706	32.7	46	+0.4	+136
					+40		52.3	+0.8	1806	34.5	10	-0.6	-56					
					+44		52.7	+1.2						1749	33.2	7	+0.5	+43
					+46		52.7	+1.2	1811	34.4	6	+0.4	+5					
					+53		53.2	+1.7						1657	31.1	9	+0.5	-92

See bottom of Table Ia for details.

• = Only two urine specimens available. Because of poor checks, both values have been included, although the low value is more likely erroneous.
= This value so far out of line with other K^{42} studies on this patient that it has not been used in calculations.
◎ = Represents the difference between the first and third K^{42} studies.

both, either, or neither systolic and diastolic pressures, respectively. Statistically significant declines in both systolic and diastolic pressures were shown by 3 of the 11 patients following this degree of sodium restriction, a fraction that approximates our experience with a similar diet during the past six years, as well as the experience of others (13–15). Eight of these patients had a decrease in TENa of 3 S.D. or more during the low Na phase and were divided without relation to the effects on blood pressure. This was noted earlier (1).

The values obtained for TEK on all patients were in agreement with those reported for normal males (3) and females (12). Among the 11 patients limitation of Na was followed by a statistically significant change in TEK in only two subjects, both males: Mon (Table Ia) and R (Table Ic). In the first instance there was an increase of 469 mEq. in TEK in association with a decline in blood pressure, and in the second there was a fall in TEK of 537 mEq. with no change in blood pressure. The remaining nine patients showed variable and modest responses in TEK which differed somewhat between the two groups. TEK in Group I was found to vary in minor degree about the control values, except for patient R as noted. By contrast, a fairly uniform reaction occurred in the members of Group II: 37 days after Na restriction TEK was elevated over control levels in all five patients. The increase exceeded 3 S.D. in only one patient (Mon, Table Ia) on a weight and sex basis, but the increases were all

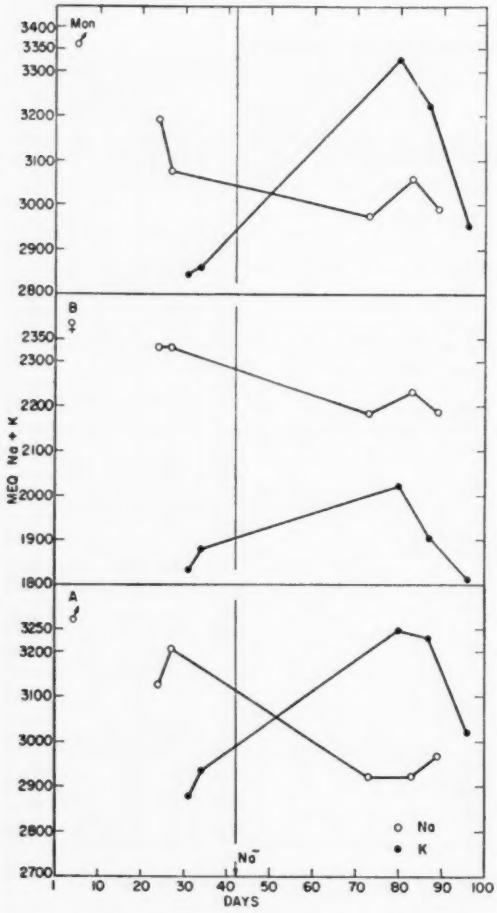

FIG. 1. TENA AND TEK BEFORE AND AFTER NA RESTRICTION IN THREE PATIENTS

The vertical line on abscissa indicates day on which added Na (180 mEq.) was omitted from diet.

similar in magnitude (+ 136 to + 469 mEq.). Recheck of TEK 44 and again 53 days after Na restriction revealed that the initial elevation was not permanent for by the 53rd day, the data obtained were in substantial agreement with those found during the control period. In Figure 1, the values on several representative patients of Group II have been plotted against time from which it will be seen that the decline in TEK was gradual. This suggested that the initial elevation noted after Na restriction represented a physiologic response rather than a methodological aberration. Furthermore, from the characteristic urinary patterns shown in Figure 2, it may be seen that Na limitation had no apparent effect on K excretion. This confirms earlier observations (1). Repeated measurements were made of serum Na and K throughout the course of the study and showed no significant deviations during either phase of the regimen.

INTERPRETATION

In confirmation of earlier work (1), these studies indicate that sodium restriction does not act by simply "salting out" the organism since desalting, at least as measured by TENa, bore no relationship to the therapeutic effect in these patients. In view of the evidence (16) that in some patients TENa can decrease or increase unrelated to net balance of sodium, the possibility must be considered that TENa may not accurately mirror any desalting which does occur. However, calculation of the net sodium balance in this group by measurement of total urinary sodium assuming a daily stool output of 1.5 mEq. per day (1) and an intake of 6 mEq. per day, failed to show that in those with the greatest net loss, the most significant declines in blood pressure occurred.

It had been found earlier (1) in four patients subjected to a simliar regimen that loss of exchangeable sodium uniformly exceeded the weight loss which should have occurred if the sodium came from extracellular fluid (ECF). Therefore, the hypothesis was proposed that failure to lose an equivalent amount of weight along with the sodium was due to a movement of water from an extracellular to an intracellular position. Analysis of the data from these 11 patients relative to this point is shown in detail in the accompanying table

FIG. 2. URINARY EXCRETION OF NA AND K BEFORE AND AFTER NA RESTRICTION

The second vertical line on patient P indicates point at which NaCl again was *added* to diet; this patient also received a total of 400 mgm. Cortisone, I.M., on 15 and 16 July, in association with studies not pertinent to the present paper. The studies on the group as a whole had been completed at this time; the effects on Na and K excretion are readily visible. Note that ordinates are logarithmic.

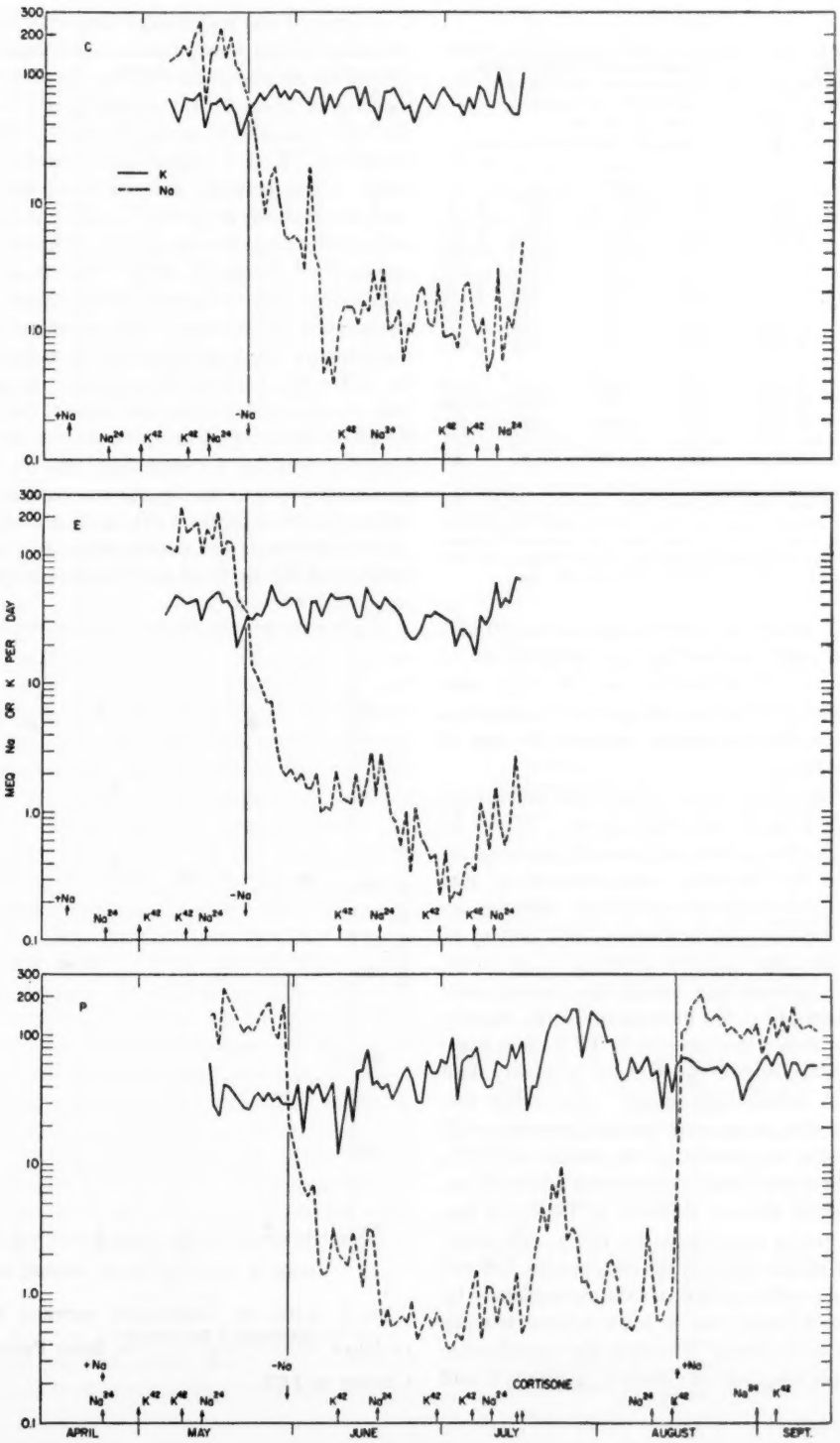
C
E
P
K
Na
MEQ Na OR K PER DAY
300
200
100
10
1.0
0.1
+Na
-Na
Na24
K42
CORTISONE
APRIL
MAY
JUNE
JULY
AUGUST
SEPT.

FIG. 2.

TABLE II

Comparison of determined TENa difference after Na restriction and theoretical Na equivalent of the weight change, assuming that all weight changes were due to variations in ECF associated with withdrawal or addition (P) of Na *

	Patient and No.	Δ Wt. (Kg.)	E.C.F. equiv. (A) (Kg.×140)	Δ TENa (B)	B−A
Group I	P 5424	−4.0	−560	−389	+171
		+1.0	+140	+330	+190
	U 5348	−0.8	−112	−117	− 5
	C 5420	−1.0	−140	−123	+ 17
	E 5274	−2.0	−280	−178	+102
	M 5283	−0.2	− 28	−152	−124
	R 5303	−4.0	−560	+124	+684
Group II	A 5455	−1.2	−168	−285	−117
	B 5504	+0.7	+ 98	−148	−246
	L 5458	−1.5	−210	−384	−174
	Mon 5472	+0.3	+ 42	−101	−143
	S 5268	+0.9	+126	+ 17	−109

* These values were obtained from the last TENa determination before, and the first TENa after, restriction of Na in the diet. The second value for patient P represents the difference between the last TENa before and the first TENa after, the *re-addition* of Na in the diet.

(Table II) which includes changes in weight and TENa recorded between the last measurement of TENa *before* Na restriction and the first such measurement *after* restriction (patient P includes a period of sodium restriction followed by one of sodium addition).

The present data neither wholly confirm nor refute the hypothesis proposed above. It will be seen that the five patients in Group II are in agreement with the proposed interpretation in that weight loss fell short of equivalence with the decrease in TENa. But in Group I only patient M followed the same pattern although P, upon re-adding Na, gained less weight than would have been predicted from her increase in TENa, thereby suggesting that if the increase in TENa was associated with an increase in ECF, the necessary fluid came from intracellular water. The other five patients in this group were not in agreement with the hypothesis suggested. In two, decline in TENa and weight were virtually equivalent while the remaining three showed declines in TENa of less than the weight equivalence by 102 to 684 mEq. Na. It is of interest that the two groups differed by such an extent and it is not simply explained by the effects of weight loss with the relative absence of such loss in Group II versus the considerable loss in some members of Group I: patients U and C in Group I lost insignificant amounts of weight, yet failed to lose more than an equivalent amount of sodium measured by TENa. Two of the five patients in Group II lost slightly greater amounts of weight than did U and C, yet had an associated decline in TENa of significantly greater equivalence. Parenthetically, a normal male that underwent the regimen in Group II, and who forms the subject of a separate report (16) behaved like the members of Group I rather than those in his own group; this suggested that changes in environmental temperature, diet, or unknown technical factors could not be called in to account for the difference between the groups. It seems at least as reasonable to interpret some of the changes in TENa as being fluctuations in the amount of body Na available for exchange with the isotope, presumably due to that in the reservoir contained in bone (17). Sjögren (18) made a similar suggestion after treating a patient with rheumatoid arthritis with 20 mg. DCA for 60 days and observing

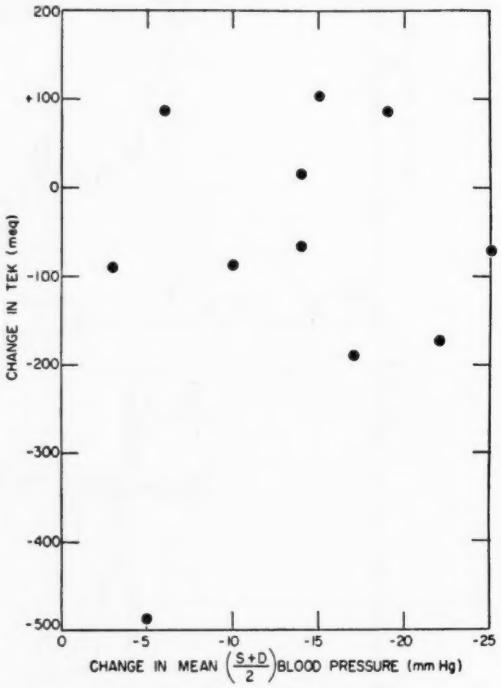

FIG. 3. LACK OF CORRELATION BETWEEN CHANGES IN MEAN $\frac{(\text{SYSTOLIC} + \text{DIASTOLIC})}{2}$ BLOOD PRESSURE AND CHANGE IN TEK

TABLE III

Summary of changes in blood pressure, TENa and TEK

Patient	Significant* decrease in B.P.		Significant decrease in TENa	Significant change (+ or −) in TEK
	Syst.	Diast.		
C	+	+	+	0
A	+	+	+	0
Mon	+	+	0	+
E	+	0	+	0
P	+	0	+	0
B	+	0	+	0
L	0	+	+	0
Mor	0	0	+	0
R	0	0	0	−
U	0	0	+	0
S	0	0	0	0

* Statistically significant.

an increase of 700 mEq. in TENa in the presence of a net Na loss of 330 mEq.

The data do not suggest that drastic limitation of Na intake acts by effects on K, at least as measured by TEK. The frank lack of correlation between blood pressure changes and TEK is shown in Figure 3. Further, as indicated in Table III, there seemed to be no relationship between changes in TEK, and those in TENa. The types of measurements used are, of course, relatively gross and it is possible that while no constant effect on K metabolism was found, fine changes were effected by the regimen but were obscured by the daily variations in K intake and the magnitude of the K pool. No explanation is presently available for the transient rise of TEK found in Group II and not in Group I. With Na restriction, it is known that K largely replaces Na as the dominant ion in sweat (5). It is therefore possible that the warm weather associated with the last half of the period in which Group I was studied could have led to K loss of considerable degree with resulting reflection upon TEK; and it is true that in this group as a whole, TEK declined slightly over control values.

The weight changes failed to account for variations in TENa as mentioned earlier; this was also true for TEK as indicated by data in columns 18 and 19, Tables Ia, Ib, and Ic. It is reasonable to suggest that here, too, the variability found is accounted for by changes due to the bone reservoir of K (17).

SUMMARY

1. A series of 11 patients with essential hypertension has been studied before and after restriction of Na intake to approximately 6 mEq. per day.

2. After restriction of Na intake, no correlations were found between changes in blood pressure and either total exchangeable sodium or potassium.

3. It was suggested that some of the variations which occurred in TENa and TEK were due to fluctuations in the amount of body sodium and potassium made available for exchange with the isotopes, presumably due to the reservoir of these elements contained in bone.

ACKNOWLEDGMENTS

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ARTERY WALL ELECTROLYTES IN RENAL AND DCA HYPERTENSION [1]

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PART I

Renal Hypertension

In an earlier study it was found that the renal artery of necropsied hypertensive subjects contained abnormally large amounts of sodium and water (1). The present study deals with the electrolyte composition of the aorta in rats with renal hypertension. After these aortas have been stripped of adventitia, at least two-fifths of the total area in tissue sections is occupied by smooth muscle cells. The remainder is largely connective tissue. The rat aorta thus provides for analysis a tissue rich in arterial smooth muscle. Moreover, the smooth muscle cells in the aorta are continuous with and have many of the pharmacological reactions of similar cells in the smaller arteries and arterioles (2). It seemed likely that any chemical alteration occurring in the aorta would give a clue to similar changes in the smooth muscle of arterioles. Technical difficulties have so far prevented us from analyzing the electrolyte content in the walls of arterioles.

METHODS

Hypertension was produced by compressing one kidney of 250 gm. male rats with a figure-of-eight ligature and removing the other kidney six days later. All the rats were fed rat chow [3] (.3 per cent sodium and 1.2 per cent potassium) and water *ad lib.* Eight weeks or longer after the second operation, blood pressure was determined and the rats were sacrificed for tissue analysis.

The aortas were dissected, stripped of adventitia, blotted free of blood, and placed in weighing bottles. In one series of rats, tissues were dried, defatted, and extracted with 0.75N HNO_3 according to the method of Lowry and Hastings (3). Sodium and potassium concentrations in the extract were determined with a Weichselbaum flame photometer. Chloride was determined by the potentiometric method of Kolthoff and Kuroda (4). In using this method, standard chloride solutions were used to bracket the unknown solutions. In another series of rats the tissues were dried and defatted as above, and extracted with 10 per cent trichloroacetic acid. Magnesium concentration was determined in this extract using the titan yellow method of Kunkel, Pearson, and Schweigert (5). Polyvinyl alcohol (Elvanol 51–05) was used to prevent precipitation of the MgOH-titan yellow complex.

Sera were analyzed for water, sodium, and potassium by the same methods. Blood urea was determined by a standard urease-nesslerization method.

Mean blood pressures were determined under ether anesthesia by direct puncture of the abdominal aorta below the level of the renal arteries. It was found that the depth of ether anesthesia could be varied considerably without changing the mean blood pressure. When the breathing of the rat indicated the proper plane of anesthesia, the blood pressure was read on the mercury manometer. This method gives very consistent results on normal rats. The average mean blood pressure of 26 normal rats was 106.7 mm. Hg with a standard deviation of only 8.4 mm. Hg.

All the normal unoperated rats had mean aortic blood pressures below 118 mm. Hg. Many of the rats underwent the kidney operations without ever developing significant hypertension. All operated rats with mean blood pressures below 121 mm. Hg were placed in the "operated normotensive" category. The average blood pressure of this group was 110 mm. Hg. Operated rats with mean blood pressures between 135 and 160 mm. Hg were considered to have moderate hypertension. Rats with mean blood pressures between 160 and 205 mm. Hg were classified as severely hypertensive.

RESULTS

With regard to sodium content (Table I), the aortas of the normal rats and the operated normotensives were not significantly different. However, the moderate hypertensive rats had a 6 per cent higher sodium content than the operated normotensives. The severe hypertensives had a 16 per cent higher sodium content than the operated normotensives. The differences between these three groups are significant.

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[2] This work was done during the tenure of an Established Investigatorship of the American Heart Association.

[3] Obtained from Uncle Johnny Mills, Houston, Texas.

TABLE I

Sodium, potassium, and chloride content of the aorta (mEq. per 100 gm. of dry fat-free aorta solids)

Groups of rats	No. of rats	Total Na*	Calculated extracellular Na*	Total Cl*	Total K*
Severe hypertensives	5	39.6 ± .9	34.2	25.4 ± .8	13.5 ± .6
Moderate hypertensives	11	36.2 ± .7	32.3	24.7 ± .4	11.3 ± .4
Operated normotensives	16	34.2 ± .7	32.5	26.0 ± .4	11.4 ± .3
Normal rats	12	35.8 ± .5	31.5	25.4 ± .9	9.5 ± .1
DCA hypertensives	6	43.5 ± 1.5	34.3	26.8 ± .4	10.9 ± .4

* Group mean.
± indicates standard error of the mean.

The chloride content of the aortas (Table I) was not significantly abnormal in any of these groups. Moreover, the chloride concentration in plasma was fairly similar for all groups. If one assumes that all of the chloride in the aorta is in the extracellular fluid in the same concentration as in an ultrafiltrate of plasma, then the amount of extracellular fluid and extracellular sodium in these aortas per 100 gm. of dry fat-free solids can be calculated by the method of Hastings and Eichelberger (6). The calculated extracellular fluid per 100 gm. of aorta solids is 228.5 gm. in the operated normotensive rats, 224.8 gm. in the moderate hypertensive group, and 234.8 gm. in the severe hypertensive group. The calculated extracellular sodium per 100 gm. of dry solids is 32.5 mEq. in the operated normotensive rats, 32.3 mEq. in the moderate hypertensive rats, and 34.2 mEq. in the severly hypertensive rats (Table I). From these calculations, it is seen that the increased Na concentration in the aortas of the moderate hypertensive rats compared to the operated normotensive rats cannot be explained even in part as an increased in extracellular sodium. The aortas from the severely hypertensive rats do have 1.7 more milliequivalents of calculated extracellular sodium per 100 gm. of solids when compared to the aortas of operated normotensive rats. However, this increase in calculated extracellular sodium accounts for only 31 per cent of the total increase in sodium content present in the aortas of the severely hypertensive rats when compared with the operated normotensive group. Considering these calculations, it seems unlikely that the increased sodium concentration in the aortas of the hypertensive rats is explainable as an increase in the proportion of extracellular fluid.

TABLE II

Magnesium content of aorta (mEq. per 100 gm. of dry fat-free solids)

	No. of rats	Group mean ± S.E. of the mean
Normal rats	8	4.61 ± .19
Operated normotensives	10	4.33 ± .16
Hypertensives	14	4.02 ± .12

The aorta potassium content (Table I) in the operated normotensive rats was 20 per cent higher than that present in the aortas of normal rats. The moderate hypertensive group showed about this same value. The severe hypertensive group had an aorta potassium content 18 per cent above that in the operated normotensive group. These differences are highly significant.

TABLE III

Serum concentrations of sodium, potassium, and chloride in milliequivalents per liter of serum

	No. of rats	Na*	K*	Cl*
Severe hypertensives	5	145.7 ± 2.3	6.9 ± .7	98.6 ± 1.4
Moderate hypertensives	11	143.7 ± 1.3	5.9 ± .4	104.7 ± .6
Operated normotensives	16	142.3 ± .8	6.2 ± .15	103.5 ± .5
Normal rats	12	143.1 ± 1.0	5.7 ± .2	105.1 ± .8
DCA hypertensives	6	145.5 ± 1.3	4.3 ± .2	103.6 ± 1.7

* Group mean.
± indicates standard error of the mean.

Table II shows the results of magnesium analyses on the aortas in a smaller series of rats. All hypertensive rats were combined in one group with an average blood pressure of 145 mm. Hg. Blood pressures in this group ranged from 127 to 205 mm. Hg. The aortas in this hypertensive group showed a magnesium content 13 per cent lower than normal, a significant difference. The severe hypertensives were no lower than moderate hypertensives.

These changes in the aortas of hypertensive rats occurred whether or not azotemia was present.

Table III gives the average serum concentrations for sodium, chloride, and potassium. The severe hypertensives show some increase in serum potassium concentration as well as a 6 per cent decrease in chloride concentration. The lowered chloride concentration is probably due in the main to uremic acidosis in two of the rats in this group.

DISCUSSION

These data indicate that the composition of the aorta becomes altered in rats with renal hypertension. The changes in the aorta are fairly specific in that brain and muscle tissue in the same rats showed relatively insignificant changes in sodium, potassium, or magnesium composition that can be correlated with hypertension (7). Moreover, the increased sodium concentration in the aortas of the hypertensive rats cannot be accounted for merely as an increase in the proportion of extracellular fluid, as mentioned. The intracellular compartment (mainly vascular smooth muscle cells) remains the most likely part of the aorta responsible for the observed changes in composition that are related to hypertension.

The fact that the potassium content increased while Mg content decreased in the aortas from hypertensive rats is unusual for muscle tissues. In skeletal muscle the ratio of these two important intracellular cations usually remains constant even under quite varying conditions (8).

We are unable to say whether or not these chemical abnormalities of the aorta play an important role in the pathogenesis of renal hypertension. However, there are several interesting relationships which suggest that they might.

The changes in the composition of the aorta associated with hypertension may be fundamentally related to the hypertensive process in a causal way or may be a result of the hypertension. An increase in lateral pressure in the aorta associated with hypertension would have the effect of ultrafiltering more extracellular fluid into the aorta wall. However, the rate of egress of this fluid into the capillaries and lymphatics of the adventitia seems to keep pace with the faster introduction of fluid, since there is no sizeable increase in the proportion of extracellular fluid in the aortas of hypertensive rats. Moreover, we have measured the water content in the aorta tissue above and below a coarctation of the aorta in five human cases, and the upper segment which is under a higher arterial pressure has the same water content as the lower segment.

The compositional changes in the aorta might be the result of a contraction of smooth muscle cells. This possibility is under investigation at the present time. Depolarization may induce electrolyte changes in these cells which in turn influence the state of contraction of actomyosin. It is also possible that the compositional changes in the aorta instead of resulting from smooth muscle contraction may either make the muscle cells more sensitive to depolarization or might induce a stronger contraction for a given degree of depolarization. Whatever mechanism is operating, the altered composition of electrolytes could conceivably have important effects on the tension of arterial smooth muscle. First, if total intracellular cation concentrations were altered, there would be changes in the membrane potential. Such changes in membrane potential have an important influence on actomyosin kinetics. It has been shown in the frog heart that a high membrane potential tends to keep the actomyosin uncontracted. Conversely, a lowered membrane potential favors contraction (9). It has also been shown that arterial strips with a lowered membrane potential are more sensitive to nor-epinephrine (2).

Secondly, it is an equally pertinent observation that actomyosin-ATP kinetics in all types of muscle are markedly influenced by the intracellular ionic environment. As little as a 3 per cent change in ion composition can mean the difference between an actomyosin contraction with maximal tension and a contraction that develops no tension (9). The amount of intracellular potassium and sodium ions in relation to intracellular solids is more im-

portant to the development of tension than the concentration of these ions in intracellular water (9). Intracellular magnesium ion content is also highly important in relation to the contraction of actomyosin as well as to its relaxation (10, 11).

Such an effect of the ions on smooth muscle membrane or actomyosin may account for Wiggers' observation that the large arteries in hypertensive patients showed a definite decrease in elasticity even in the absence of atherosclerosis or old age (12).

It is known that diets low in either sodium or potassium cause a reduction in blood pressure in rats with renal hypertension (13, 14). These dietary effects are also present in hypertensive man (15, 16). It is possible that these diets lower blood pressure by reducing the elevated sodium or potassium in the arteries of hypertensives to a more normal level. This possibility is now under investigation.

It is not known whether adrenal cortical hormones participate in the arterial electrolyte changes associated with renal hypertension. In another part of this study, it was found that the aortas of rats with DCA hypertension show significant increases in sodium and potassium concentration, a pattern similar to that seen in the aortas of rats with renal hypertension.

As seen in the "operated normotensive" group, renal damage resulting from the operation evidently can produce an elevation of potassium concentration in the aorta, part of the chemical "lesion" of hypertension, without causing a significant rise of the blood pressure. This elevation was not necessarily associated with azotemia since it occurred in operated normotensive rats with a normal blood urea nitrogen level.

PART II

Desoxycorticosterone Hypertension

Large doses of desoxycorticosterone acetate (DCA) in combination with a high sodium intake will produce hypertension in man as well as in many lower mammals. This rise in blood pressure can be completely prevented by diets very low in sodium (17) or potassium (18) content. The hypertension accompanying DCA is increasingly severe as larger amounts of sodium are included in the diet. In view of the great influence of dietary sodium and potassium on this type of hypertension, it seemed of interest to determine the sodium and potassium concentrations of arteries in DCA hypertensive rats and compare them with the findings in normal rats.

METHODS

Hypertension due to DCA was produced in 250 gm. male rats by injecting subcutaneously 5 mgm. of DCA ("Percorten")[4] every day for six weeks. During this period the rats were allowed to drink only a 1 per cent NaCl solution. There were no operations or manipulations involving the kidney. During the entire injection period, the rats appeared healthy and active. At the end of the six week injection period, the rats were exsanguinated and samples of aorta, cerebrum, and hamstring muscles were obtained for chemical analysis. The aortas were stripped of adventitia, and as much connective tissue as possible was trimmed from the skeletal muscle samples prior to analysis.

Methods for determining blood pressure and for the chemical analyses are described in Part I of this paper. Chloride in brain and muscle was determined by the Volhard method (3). A blood pressure of 124 mm. Hg is two standard deviations above the mean for the normal rats. Any blood pressure above this level was considered hypertensive. The six rats which developed DCA hypertension had blood pressures ranging from 125 to 158 mm. Hg with a group average of 136 mm. Hg. These rats were compared with the group of normal rats similar in age and sex mentioned in Part I of this paper. Both normal and hypertensive rats were fed a rat chow containing .3 per cent sodium and 1.2 per cent potassium. The normal rats were allowed to drink water *ad lib.*

RESULTS AND DISCUSSION

The content of sodium and potassium in the aortas is given in Table I. The aortas of the DCA hypertensive rats had a 22 per cent greater sodium content and a 15 per cent greater potassium content (per 100 gm. DFFS) than the aortas of normal rats. These differences are statistically significant.

The DCA hypertensive aortas also had a 5 per cent greater chloride concentration, but this was not a significant difference.

Table III shows the serum concentrations of Na, K, and Cl in the normal and DCA hypertensive rats. As would be expected from many reports the DCA hypertensives have a slightly higher than

[4] Percorten was kindly furnished by Dr. Ernst Oppenheimer of the Ciba Pharmaceutical Company.

normal serum sodium concentration and a lower potassium and chloride concentration.

If one assumes that all the chloride in the aorta is in the extracellular fluid, then one can calculate the amount of extracellular fluid and extracellular sodium per 100 gm. of dry fat-free solids using the system of Hastings and Eichelberger (6). In the aortas of DCA hypertensive rats there were 236 gm. of extracellular fluid per 100 gm. of solids compared to 220 gm. in the normal aortas. The aortas of the DCA rats had a calculated 34.3 mEq. of extracellular sodium per 100 gm. of dry solids, compared to 31.5 mEq. of extracellular Na in the normal aortas (Table I). The DCA hypertensive aortas had 7.7 more mEq. of total Na per 100 gm. of solids than the normal aortas; 2.9 of these extra milliequivalents can be explained by an increase in extracellular Na in the DCA aortas. The other 4.8 mEq. of increased sodium content in the DCA aortas is probably an increase in intracellular sodium.

The implications of altered intracellular sodium and potassium with respect to the tonus of arterial smooth muscle have been discussed in Part I of this paper.

It is of interest that the concentrations of sodium and potassium in the aorta are increased in both renal and DCA hypertension. It is too early to tell whether this pattern is a fundamental chemical "lesion" that accompanies all types of experimental hypertension in the rat.

PART III

Comparison of the Electrolyte Changes in Artery, Brain, and Skeletal Muscle Produced by Desoxycorticosterone and Salt

After the administration of large doses of desoxycorticosterone acetate (DCA) in conjunction with a large sodium intake, most mammals show a positive sodium balance and a negative potassium balance. This is reflected in slight increases in serum sodium concentration and decreases in serum potassium concentration. It is also reflected in an increased sodium concentration and a decreased potassium concentration in skeletal muscle (19). It is not clear, however, that tissues other than skeletal muscle behave in a similar manner. This is especially important in regard to arteries, inasmuch as the compositional changes in skeletal muscle induced by DCA have been assumed to occur in the arteries as well. The changes in the electrolyte composition of brain, skeletal muscle, and aorta under the influence of DCA and salt were therefore studied to determine to what extent the responses of different tissues are the same as those of skeletal muscle. These analyses were carried out on the rats made hypertensive with DCA as well as the normal control rats that are described in Part II of this paper.

RESULTS AND DISCUSSION

The tissue composition of skeletal muscle in the DCA treated rats is presented in Table IV. Confirming previous reports, the sodium content of muscle increased from 8.2 mEq. (per 100 gm. DFFS) in the normal group to 14.1 mEq. in the DCA group while the potassium content dropped from 47.5 mEq. (per 100 gm. DFFS) in the normal group to 39.5 mEq. in the DCA group. The chloride content of skeletal muscle rose from 4.1 mEq. per 100 gm. DFFS in the normal rats to 5.0 mEq. in the DCA rats. These differences are highly significant. The water content was almost exactly the same in the two groups.

Assuming that all the chloride in whole skeletal muscle is in the extracellular phase at the same concentration as in an ultrafiltrate of plasma, the partition of water, sodium, and potassium between the extracellular and intracellular phases can be estimated using the methods of Hastings and Eichelberger (6). The intracellular phase lost 8 mEq. of potassium per 100 gm. DFFS while gaining 4.6 mEq. of sodium. In terms of concentration per liter of cell water, sodium increased 17 mEq. per liter above normal and potassium fell 24.3 mEq. per liter below normal in the DCA rats (Table IV).

Analysis of brain in the DCA group gave somewhat different results (Table V). The mEq. of sodium per 100 gm. DFFS increased from 23.9 in the normal rats to 25.4 in the rats receiving salt and DCA. Chloride (per 100 gm. DFFS) also increased from 21.2 mEq. in the normal rats to 21.8 mEq. in the DCA group. This is a much smaller increase in sodium than occurred in skeletal muscle. Furthermore, the brain potassium content per 100 gm. DFFS increased 10 per cent above normal in the DCA rats instead of showing

TABLE IV

Water and electrolyte composition of skeletal muscle *

	No. of rats	Direct data Na† (*mEq.*)	K† (*mEq.*)	Cl† (*mEq.*)	H_2O_t† (*gm.*)	Derived data H_2O_E† (*gm.*)	H_2O_I† (*gm.*)	$(Na)_I$† (*mEq.*)	$(K)_I$† (*mEq.*)	$[Na]_I$ (*mEq.*)	$[K]_I$ (*mEq.*)
Normal rats	12	8.2±.15	47.5±.9	4.1±.1	318.4±4.2	35.6	282.8	3.1	47.3	11.0	167.5
DCA treated rats	6	14.1±.5	39.5±.8	5.0±.1	318.8±1.9	44.0	274.8	7.7	39.3	28.0	143.2
% difference		+72%	−17%	+22%	+.1%	+24%	−3%	+148%	−17%	+155%	−14.5%
p value		<.001	<.001	<.001	.9						

* ± indicates standard error of the mean.
H_2O_t total water per 100 gm. of dry fat-free muscle solids.
H_2O_E extracellular water per 100 gm. of dry fat-free muscle solids.
H_2O_I intracellular water per 100 gm. of dry fat-free muscle solids.
$(\ )_I$ mEq. of intracellular cation per 100 gm. of dry fat-free muscle solids.
$[\ ]_I$ mEq. of intracellular cation per Kg. of cell water.
† Per 100 gm. of dry fat-free muscle solids.

the decrease that is characteristic of skeletal muscle. The DCA group had an average of 51.8 mEq. of potassium per 100 gm. of DFFS compared to 47.1 mEq. in the normal group. All these differences between the normal and DCA groups were statistically significant. Water content was not significantly different in the two groups.

If the "chloride space" calculations are applied to brain in the same manner as was used with skeletal muscle, negative values for intracellular sodium are obtained. This is probably explained by the fact that brain cells are bathed by a fluid with the composition of cerebrospinal fluid rather than an ultrafiltrate of plasma. The concentration of chloride in cerebrospinal fluid exceeds that in an ultrafiltrate of plasma by about 12 mEq. per liter. Using this assumption the partition of water, sodium, and potassium between extra- and intracellular phases has been calculated and is shown in Table V. The calculations are the same as those applied to skeletal muscle except that the concentration of chloride in brain extracellular fluid was assumed to be 12 mEq. per liter more than in an ultrafiltrate of plasma. By these methods, the calculated intracellular sodium is zero in normal brain tissue and it increases by an insignificant amount (.06 mEq. per 100 gm. DFFS) in the brain tissue of the DCA group. The intracellular potassium of brain per 100 gm. DFFS increases 10 per cent above normal. Thus the intracellular phase in the DCA rats gains 4.8 mEq. of potassium per 100 gm. DFFS and keeps about the same content of sodium, while losing 7.6 gm. of water. Consequently, the calculated concentration of potassium per Kg. of brain cell water increases from 119 mEq. per Kg. in the normal group to 134 mEq. per Kg. in the DCA group.

As mentioned in Part II of this paper, the aorta shows still another pattern of change under the influence of DCA and salt. In the aorta both sodium and potassium (per 100 gm. DFFS) are increased above normal levels in the DCA group.

TABLE V

*Water and electrolyte composition of brain**

	No. of rats	Direct data Na† (*mEq.*)	K† (*mEq.*)	Cl† (*mEq.*)	H_2O_t† (*gm.*)	Derived data H_2O_E† (*gm.*)	H_2O_I† (*gm.*)	$(Na)_I$† (*mEq.*)	$(K)_I$† (*mEq.*)	$[Na]_I$ (*mEq.*)	$[K]_I$ (*mEq.*)
Normal rats	12	23.9 ±.3	47.1±1.0	21.2±.2	555.6±3.0	166.8	388.8	0	46.3	0	119.0
DCA rats	6	25.35±.7	51.8±1.2	21.8±.2	555.0±4.5	173.8	381.2	.06	51.1	0.16	134.0
% difference		+6%	+10%	+3%	−.1%	+4%	−2%		+10%		+13%
p value		.04	<.001	.02	.9						

* ± indicates standard error of the mean.
H_2O_t total water per 100 gm. of dry fat-free brain solids.
H_2O_E extracellular water per 100 gm. of dry fat-free brain solids.
H_2O_I intracellular water per 100 gm. of dry fat-free brain solids.
$(\ )_I$ mEq. of intracellular cation per 100 gm. of dry fat-free brain solids.
$[\ ]_I$ mEq. of intracellular cation per Kg. of cell water.
† Per 100 gm. of dry fat-free brain solids.

The results reveal that large doses of DCA combined with a high sodium intake produce considerable alterations in tissue electrolyte concentrations. The most significant conclusion, however, is that the various tissues do not respond in like manner to the administration of DCA and salt. Skeletal muscle fibers gain in sodium and lose potassium. Brain cells gain considerable potassium while gaining only insignificant amounts of sodium. The smooth muscle of the aorta gains not only potassium but sodium as well. Thus there is no uniform biological response of all types of cells to DCA administration, and each type of tissue cell must be considered separately.

This is also true for cortisone. Streeten and Solomon have found increases in the potassium concentration of red cells after cortisone administration (*20*), while Seldin, Welt, and Cort have shown that potassium concentration in skeletal muscle decreases under the influence of cortisone (*21*).

In regard to DCA hypertension, these results indicate that one cannot make valid inferences concerning the effect of DCA and salt on the electrolyte composition of arteries by analyzing skeletal muscle.

SUMMARY OF PARTS I, II, AND III

1. The aortas of rats with renal hypertension have both a higher sodium and a higher potassium concentration than the aortas of rats which have undergone the same type of kidney operation but remain normotensive.

2. The aortas of these "operated normotensive" rats have a higher potassium concentration than the aortas of normal rats.

3. In a smaller series of rats, the aortas of renal hypertensive rats have a 13 per cent lower magnesium concentration than normal rats.

4. The aortas of DCA hypertensive rats also have both a higher sodium and a higher potassium concentration than seen in normal aortas. Thus, the aortas have the same type of chemical alterations in both renal and DCA hypertension.

5. From calculations of "chloride space," no more than a third of the increased sodium concentrations in the hypertensive aortas can be explained as an increase in extracellular sodium.

6. DCA greatly increases sodium and decreases potassium concentration in rat skeletal muscle. DCA causes a slight increase in brain sodium concentration, but produces a considerable increase in brain potassium concentration. DCA effects a considerable increase in both sodium and potassium concentration in rat aorta.

7. Thus, with regard to electrolyte composition, there is no uniform biological response in all types of cells to DCA administration. Each type of tissue cell must be considered separately.

8. Inferences concerning the electrolyte composition of arteries in DCA hypertension cannot be made from analyses of skeletal muscle.

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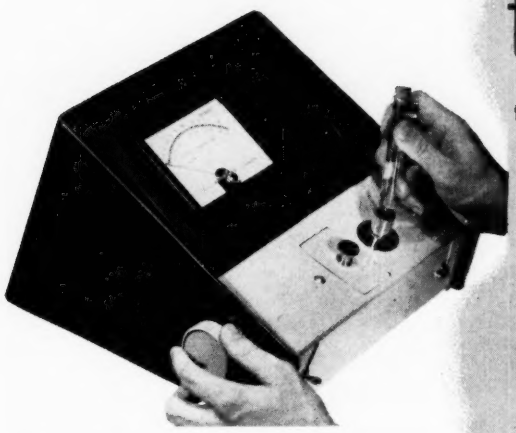

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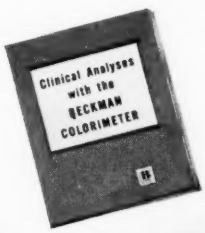

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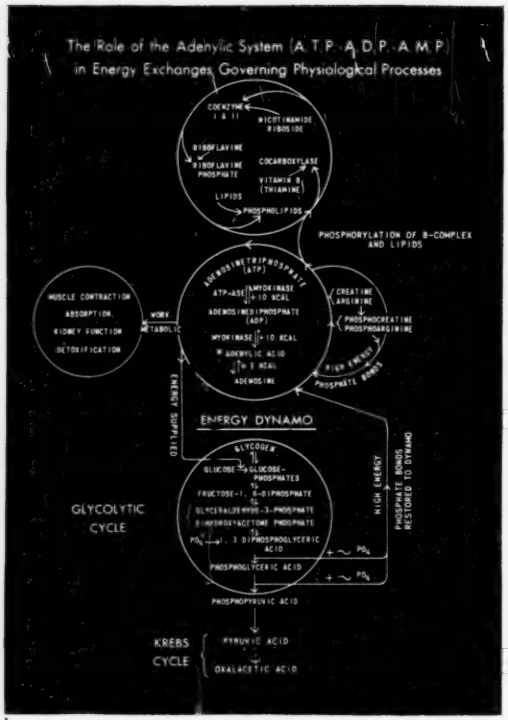

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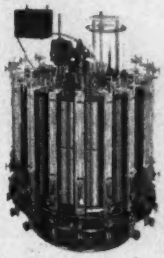

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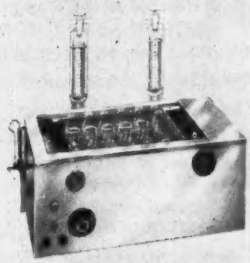

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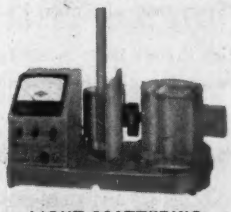

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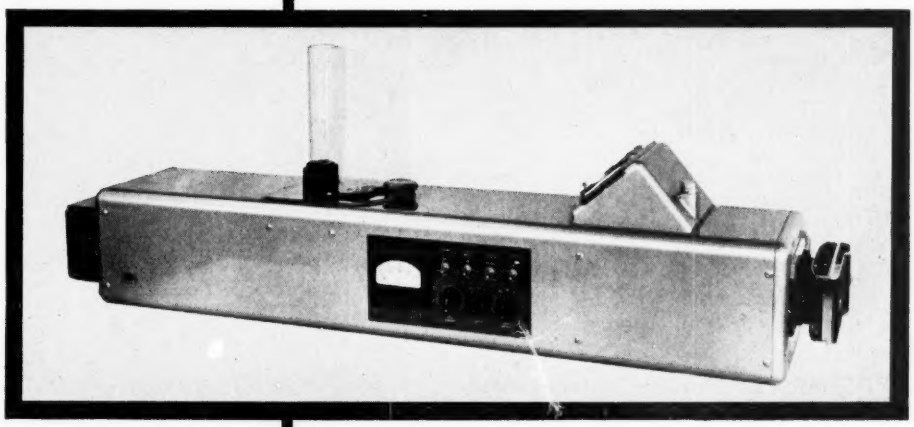

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Numerous investigations have shown that rheumatic-fever prophylaxis requires continuous control of streptococcal infection in the upper respiratory tract. Since the cost of such a long-term program may be a determining factor, studies of relatively inexpensive home therapy are of particular interest. One of these studies has been recently reported on by Tidwell,[1] speaking at the annual meeting of the American Medical Association, June, 1954.

Prophylactic medication to eradicate the beta-hemolytic streptococcus was given to 59 patients during the streptococcal season of 1952-1953. Tidwell reports that Bicillin, a highly insoluble salt of penicillin, was chosen for study because several clinical investigations had demonstrated it to be the most efficient agent for production of the extended penicillin blood levels required.

Of these 59 patients, 25 were acutely or chronically ill at the start of the program. Streptococcal pharyngitis was found in 1; congenital cardiac disease in 8; a history of rheumatic fever in 13; nephritis in 2; and asthma in 1.

THROAT CULTURES

Throat cultures were performed at the beginning of the investigation, as well as monthly or oftener throughout the course. Material for culture was obtained by swabbing the tonsils and posterior pharynx through the mouth under direct vision. The swabs were then streaked on the surface of blood agar plates. These cultures were found initially positive for beta-hemolytic streptococci in 5 patients.

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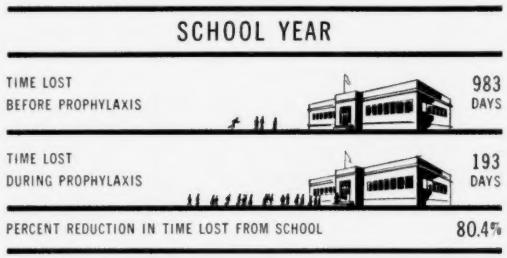
One 200,000-unit tablet of Bicillin was given daily to each patient. Some patients took the medication before breakfast, others after breakfast or before lunch, others during dinner, and some at no fixed time.

RESULTS

Tidwell relates the effectiveness of the program to the patients' school attendance. During the prophylactic course, children of school age lost only 193 days because of illness, while in the year prior to the study they had lost 983 days. School absenteeism was consequently reduced by 80.4 percent.



Of all patients, only 2 showed reappearance of the beta-hemolytic streptococcus. In the first, streptococcal breakthrough appeared after the patient had been without medication for 3 days, but the culture returned to negativity when Bicillin was resumed. In the second, the culture became negative when the dose was doubled.

No early or late suppurative or non-suppurative sequelae followed any of the infections. There was neither recurrence of rheumatic fever nor evidence of subacute bacterial endocarditis.

Of the original 59 patients, 33 are being continued on a 5-year prophylactic program. The second streptococcal season has now been completed for these patients. Tidwell reports that the beta-hemolytic streptococcus has not reappeared in any of the cultures, and that there has been no recurrence of rheumatic fever or evidence of subacute bacterial endocarditis.

1. Tidwell, R. A.: Read before Annual Meeting of A.M.A., San Francisco, Calif., June 24, 1954.

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